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14. ABSTRACT

Organophosphorus esters (OP) are highly toxic poisons used as chemical nerve agents and as pesticides. It is generally agreed that the toxicity from high dose OP exposure involves inhibition of acetyl cholinesterase. The role of other proteins in the toxicity of OP is unknown. Our hypothesis is that several proteins become modified after exposure to OP and that the biological actions of OP are not explained by inhibition of acetylcholinesterase alone. We are using mass spectrometry to identify proteins modified by exposure to OP. A new motif for OP binding is beginning to emerge from our work. We are seeing a pattern of covalent OP binding to tyrosine where the tyrosine is adjacent to an arginine or lysine. We have identified tyrosine 411 on the surface of human albumin and tyrosine 238 of human transferrin as OP-binding sites. Our results are relevant to diagnosis of OP exposure. The mass spectrometry methods we have developed are rapid and simple, but expensive. The new information from our mass spectrometry results can be used to develop antibody based dipstick assays to diagnose OP exposure.

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone; source of standard peptides for calibrating the mass spectrometer
amu	atomic mass unit
BChE	butyrylcholinesterase
CHCA	alpha-cyano 4-hydoxycinnamic acid; matrix for MALDI
CPO	chlorpyrifos oxon
DFP	diisopropylfluorophosphate
DHBA	2,5-dihydrobenzoic acid; matrix for MALDI
FP-biotin	biotin-tagged organophosphorus agent; 10-fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide
HPLC	high performance liquid chromatography
ip	intraperitoneal
LC/MS/MS	liquid chromatography coupled to tandem mass spectrometry
MALDI-TOF	matrix assisted laser desorption ionization-time of flight mass spectrometer
MS/MS	tandem mass spectrometry in which the mass of a molecule is determine in the first MS and the masses of the fragments are determined in the second MS
m/z	mass to charge ratio
OP	organophosphorus toxicant
PVDF	polyvinylidene fluoride membrane for protein blots
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TPCK	tosyl phenylalanyl chloromethyl ketone; irreversible inhibitor of chymotrypsin
UCSF	University of California San Francisco
UNMC	University of Nebraska Medical Center
Y	single letter code for tyrosine

INTRODUCTION

Organophosphorus esters (OP) are highly toxic poisons used as chemical nerve agents and as pesticides. It is generally agreed that the toxicity from high dose OP exposure involves inhibition of acetylcholinesterase (Maxwell et al., 2006). The role of other proteins in the toxicity of OP is unknown. Our hypothesis is that several proteins become modified after exposure to OP and that the biological actions of OP are not explained by inhibition of acetylcholinesterase alone. The basis for this hypothesis is our finding that a mouse treated with a biotin-tagged nerve agent analog, called FP-biotin, acquires many biotin-tagged proteins in blood. Support for our hypothesis comes from the work of toxicologists who find that poisoning symptoms are different for each OP when the dose of OP is nonlethal (Moser, 1995). For example, fenthion decreases motor activity but does not alter the tail-pinch response, whereas parathion does not lower motor activity but does decrease the tail-pinch response. If all OP reacted only with acetylcholinesterase and with no other proteins, then all OP should give the same toxic signs.

We are using mass spectrometry to identify proteins modified by exposure to OP. Until now it has been thought that the OP-reactive proteins are exclusively serine hydrolases, characterized by serine in the active site. Our work shows that this is too narrow a view, and that OP also modify proteins without an active site serine. A new motif for OP binding is beginning to emerge from our work. We are seeing a pattern of covalent OP binding to tyrosine where the tyrosine is near an arginine or lysine.

Our results are relevant to diagnosis of OP exposure. The mass spectrometry methods we have developed are rapid and simple, but expensive. The new information from our mass spectrometry results can be used to develop antibody based dipstick assays to diagnose OP exposure.

APPROVED STATEMENT OF WORK

Task 1. Identify proteins labeled by FP-biotin

1.1. Identify FP-biotin labeled proteins in human blood reported 20 April 2006

1.2. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated human protein. reported 20 October 2006

1.3. Identify FP-biotin labeled proteins in mouse blood reported 21 July 2006

1.4. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated mouse protein. reported annual report 24 December 2006

1.5. Inject mice with FP-biotin and identify the FP-biotinylated proteins in mouse blood. reported July 20, 2007

Task 2. Identify proteins labeled by DFP

2.1. Label pure human butyrylcholinesterase with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation. report for 20 Jan 2007 sent 7 March 2007

2.2. Label pure human albumin with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation. reported July 21, 2006; Li et al paper

2.3. Label pure bovine trypsin with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation. reported July 20, 2007

2.4. Identify the proteins in human blood that bind DFP. final report 20 Nov 2007

2.5. Identify the proteins in mouse blood that bind DFP. reported April 20, 2007

2.6. Inject mice with DFP and identify the DFP-labeled proteins in mouse blood. reported July 20, 2007

TASK 1

Task 1. Identify proteins labeled by FP-biotin
1.1. Identify FP-biotin labeled proteins in human blood
1.2. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated human protein.

Relation to statement of work. Results for tasks 1.1 and 1.2 are reported.

Tasks 1.1 and 1.2

OP-binding proteins in human plasma: a new motif for OP binding to tyrosine

Summary

OP binding proteins in human plasma were identified after labeling human plasma with FP-biotin. The FP-biotinylated proteins were purified on immobilized avidin beads, and separated by gel electrophoresis. Coomassie stained bands were digested with trypsin and analyzed by LC/MS/MS. Proteins were identified by analysis of fragmentation patterns using Mascot software. Thirty-one proteins were identified. Eight proteins have the consensus sequence for serine hydrolases. Three proteins, albumin, transferrin, and apolipoprotein bind OP to tyrosine. Twenty proteins have an unknown OP binding site. Samples for mass spectrometry analysis were prepared by a second method, by purifying FP-biotinylated peptides on monomeric avidin beads. This method identified FP-biotinylated peptides where the OP was covalently bound to tyrosine or serine, but did not identify the parent protein. A new motif for OP binding to tyrosine is beginning to emerge.

Introduction

Diagnosis of OP exposure relies on symptoms of cholinergic toxicity and on laboratory tests of cholinesterase activity in blood. A few specialized laboratories have gas chromatography/mass spectrometry equipment to look for OP metabolites in blood and urine. The fluoride reactivation method (Polhuijs et al., 1997; Adams et al., 2004; Degenhardt et al., 2004) recovers OP covalently attached to protein and then analyzes the released OP by gas chromatography/mass spectrometry. Another method to analyze OP exposure is

to measure the mass of the butyrylcholinesterase active site peptide (Fidder et al., 2002; Van Der Schans et al., 2004; Tsuge and Seto, 2006). The assumption in these assays is that butyrylcholinesterase in plasma and acetylcholinesterase in red blood cells are the only proteins modified by OP. It was a surprise therefore, when we treated mice with a biotin-tagged OP called FP-biotin, and found that the majority of the labeled protein was albumin (Peeples et al., 2005). Many additional proteins also became labeled. We set out to identify these additional proteins using mass spectrometry.

Materials and Methods

Materials. FP-biotin was custom synthesized in the laboratory of Dr. Charles Thompson at the University of Montana, Missoula, MT. FP-biotin was dissolved in methanol and stored at -80˚C. Immobilized avidin beads (Pierce #20219) and immobilized monomeric avidin beads (Pierce #20228) were used to purify FP-biotinylated proteins as well as FP-biotinylated peptides. Affi-gel blue gel (BioRad #153-7301) is a crosslinked agarose bead with covalently attached Cibacron Blue F3GA dye used for depletion of albumin from plasma. Proteome Partitioning Kit, ProteomeLab IgY-12 High Capacity in Spin Column format contains IgY antibodies directed against the 12 most abundant proteins in human plasma (Beckman Coulter #A24331 S0510903). Microwave Blue (Protiga, Frederick, MD #P1002-MWB) was used to stain polyacrylamide gels.

Porcine pepsin (Sigma, St. Louis, MO; P6887 from porcine gastric mucosa) was dissolved in 10 mM HCl to give a 1 mg/ml solution and stored at -80˚C. Porcine trypsin (Promega, Madison, WI; V5113 sequencing grade modified trypsin) had a concentration of 0.4 µg/µl in 50 mM acetic acid. Trypsin was stored at -80˚C. Human butyrylcholinesterase was purified from outdated human plasma (Lockridge et al., 2005). Purified human transferrin, human complement component 3, human alpha-2-macroglubin, and human alpha-1anti-trypsin were from Sigma.

Human plasma labeled with FP-biotin. 2 to 10 ml of human plasma were treated with 200 µM FP-biotin for 5 h at room temperature. Unreacted FP-biotin was removed by dialysis. A second preparation of FP-biotin labeled plasma was depleted of albumin by chromatography on a column of Affi-gel blue. A third preparation of FP-biotinylated plasma was depleted of the 12 most abundant plasma proteins by binding to antibodies in the Proteome Partitioning Kit.

Purification of FP-biotin-labeled proteins. FP-biotin-labeled proteins were purified by binding to tetrameric avidin beads, where binding is so tight that the proteins can only be released by boiling in SDS. Preparations of this type were loaded on SDS polyacrylamide gels for separation of individual FP-biotinylated proteins. FP-biotin-labeled proteins were also purified on monomeric avidin beads from which they were released with 10% acetic acid.

Purification of FP-biotin-labeled peptides. In some experiments the FP-biotin-labeled peptides rather than FP-biotin-labeled proteins were purified on monomeric avidin beads where the peptides were released with 10% acetic acid.

Digestion with trypsin or pepsin. FP-biotin labeled proteins were denatured in 8 M urea, disulfide bonds were reduced with 10 mM dithiothreitol at pH 8.0, and carbamidomethylated with 0.1 M iodoacetamide. Samples were desalted by dialysis against 10 mM ammonium bicarbonate pH 8.5. The equivalent of 200 µl plasma, containing 10 mg of protein, was digested with 0.1 mg of trypsin in 20 mM ammonium bicarbonate pH 8.5 at 37˚C for 4 to 16 h. Trypsin was inactivated with 10 mM DFP or by boiling before the sample was loaded on monomeric avidin beads.

For digestion with pepsin, the pH of the plasma sample was reduced to pH 1.8-2.1 with 1% trifluoroacetic acid. 10 mg plasma protein was digested with 0.05 mg pepsin for 2 h at 37˚C. The pepsin was inactivated by raising the pH to 8.9 before loading the digest on monomeric avidin beads.

Controls. The negative control was human plasma treated with all reagents except FP-biotin. The positive control was FP-biotinylated human butyrylcholinesterase.

Gel electrophoresis. FP-biotinylated plasma proteins were separated on SDS polyacrylamide gels or on nondenaturing polyacrylamide gels. Gels were stained with Coomassie blue or with Microwave Blue. Gel bands were cut out and prepared for digestion with trypsin as described (Peeples et al., 2005).

Protein blot. Proteins were transferred from a polyacrylamide gel to PVDF membrane and hybridized with Streptavidin Alexafluor 680 (Molecular Probes). Fluorescence intensity was captured in the Odyssey Infrared Imaging System (LiCor). This procedure visualized FP-biotinylated proteins.

MALDI-TOF mass spectrometry. All peptide samples were screened by MALDI-TOF before they were analyzed in the QTRAP mass spectrometer, because MALDI-TOF is a quick way to estimate the number of FP-biotinylated peptides and their masses. Salt-free peptides were plated on a stainless steel target plate in 1 µl aliquots, allowed to dry, and overlaid with 10 mg/ml CHCA matrix in 50% acetonitrile, 0.1% trifluoroacetic acid. Mass spectra were acquired with the Voyager-DE PRO MALDI-TOF or the MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA).

Tandem quadrupole mass spectrometry at UNMC. Tandem mass spectra (MS/MS) were acquired on a QTRAP 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) with a nano electrospray ionization source. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion

scans were obtained with collision energy of 40±5 V and nitrogen gas pressure of 4×10^{-5} Torr.

Thermo-Finnigan LTQ linear ion trap mass spectrometry at Wistar. One set of FP-biotin labeled human plasma proteins was sent to Dr. Kaye D. Speicher at the Wistar Proteomics Facility in Philadelphia, PA for identification. The FP-biotinylated proteins had been purified on tetrameric avidin beads and separated by SDS gel electrophoresis. The same set of proteins was also analyzed at UNMC on our QTRAP mass spectrometer.

Data analysis. Hundreds of extracted ion chromatograms were manually searched for the presence of the characteristic fragment ions of FP-biotin at 227, 312, 329 amu. A peptide that carries the FP-biotin tag on serine or threonine also has an ion at 591 amu (Schopfer et al., 2005). Ion masses of 227, 312, and 329 in the absence of mass 591 indicate FP-biotin is covalently bound to tyrosine. During the course of this work we have discovered an ion of mass 708.4, whose presence is a signature for FP-biotinylated tyrosine. The 708.4 mass is tyrosine covalently bound to FP-biotin. Peptides that fragmented to give ions characteristic of FP-biotin were manually sequenced from data in the MS/MS spectra.

Results

Mass spectrometry of FP-biotinylated human plasma proteins. Analysis of FP-biotin labeled proteins extracted from gel slices has tentatively identified the 31 proteins listed in Table 1.1.1. What is tentative is the interpretation that these proteins bind OP. Until we actually identify the residue that binds the OP or perform a protein blot with purified protein, we consider the identification tentative.

We are certain that the 10 proteins in Table 1.1.2 bind OP covalently. We have mass spectrometry data to prove that albumin, transferrin, and apolipoprotein bind OP on tyrosine. The 4 serine hydrolases - plasminogen, prothrombin, complement component 1 s subcomponent, and butyrylcholinesterase - bind OP on the active site serine. Three additional proteins bind FP-biotin covalently as determined from protein blots. When purified alpha-2-macroglobulin, complement component 3,and anti-trypsin proteins were treated with FP-biotin, boiled in SDS, subjected to SDS gel electrophoresis, and transferred to PVDF membrane, the blots gave an intense fluorescent signal with Streptavidin Alexafluor 680. This demonstrated that human alpha-2-macroglobulin, human complement component 3, and human anti-trypsin covalently bind FP-biotin, though the attachment site has not yet been identified.

Table 1.1.1. Preliminary identification of OP binding proteins in human plasma.

	protein	MOWSE	# peptides	MW	gi #	GXSXG
1	hemoglobin chain D	366	9	15869	1431652	-
2	hemoglobin chain C	111	3	15389	493852	-
3	serum albumin	451	13	69366	28592	-
4	inter-alpha globulin inhibitorH2	349	10	105217	55958063	-
5	inter-alpha globulin inhibitorH1	254	6	101403	4504781	-
6	alpha-2 macroglobulin	1985	51	163175	25303946	-
7	ceruloplasmin	117	4	115473	1620909	-
8	complement component 5	334	13	141723	179692	-
9	plasminogen	279	8	90496	190026	GDSGG
10	IG GI HNie	295	7	49174	229601	-
11	immunoglobulin kappa light chain VLJ region	365	7	29280	21669423	GGSGG
12	fibrinogen gamma	211	7	49450	182439	-
13	gelsonin isoform b	304	9	80641	38044288	-
14	alpha fibrinogen precursor	264	4	69767	182424	-
15	chain E, fragment double-D from fibrin	189	7	37649	28373962	-
16	fibrinogen alpha A	283	5	49398	223918	-
17	complement component 3	409	11	187046	4557385	-
18	IgG kappa chain	332	6	23419	4176418	-
19	IgM kappa IIIb SON	329	5	13765	224377	-
20	proapolipoprotein	158	4	28962	178775	-
21	fibronectin 1 isoform 6 preproprotein	3250	31	243078	47132549	GGSRG GNSNG GNSNG GLSPG
22	fibronectin homolog	470	14	272269	31873670	GGSRG GNSNG GNSNG GLSPG GNSLG
23	prothrombin	1052	10	70038	4503635	GDSGG
24	lipoprotein B100	5013	114	512880	225311	-
25	complement component C1s	677	18	76685	41393602	GDSGG
26	butyrylcholinesterase	712	18	68419	4557351	GESAG
27	transferrin	391	20	77051	15021381	GRSAG
28	Ig mu chain C region	119	9	49557	127514	-
29	Igg B12	275	10	50417	15825648	-
30	IGHA1 protein	275	7	53,377	21619010	-
31	alpha-1 antitrypsin	227	15	46,709	54695780	-

MOWSE score is the probability of a match between the experimental data and the peptide mass in the database. MOWSE scores greater than 69 are significant ($p<0.05$). # peptides is the number of peptides whose sequence matched the protein. MW is the molecular weight. gi # is the accession number. GXSXG is the consensus sequence around the active site serine.

Tyr 411 of albumin. Mass spectrometry was used to identify the amino acid to which the FP-biotin was covalently attached. Tyrosine 411 of human albumin is the attachment site for FP-biotin. Tyr 411 is also the covalent binding

site for soman, sarin, DFP, chlorpyrifos oxon and dichlorvos (Li et al., 2007). Tyr 411 is located near the surface of the albumin molecule (Ghuman et al., 2005).

Table 1.1.2. Human plasma proteins that bind FP-biotin covalently.

protein	accession #	OP binding site
albumin	gi:28592	Tyr 411
transferrin	gi:15021381	Tyr 238
apolipoprotein	gi:178775	Tyr 172
alpha-2-macroglobulin	gi:163175	
complement component 3	gi:4557385	
alpha-1 anti-trypsin	gi:54695780	
plasminogen	gi:190026	Ser 760
prothrombin	gi:4503635	Ser 568
complement component C1s	gi:41393602	Ser 195
butyrylcholinesterase	gi:4557351	Ser 198

The OP binding site is not yet known for 3 proteins in Table 1.1.2. However, we are confident that these 3 proteins bind OP covalently because protein blots of FP-biotinylated pure proteins give a strong signal when hybridized with Streptavidin Alexafluor 680.

Tyr 238 of transferrin. Tryptic peptides of pure human transferrin labeled with FP-biotin were isolated by binding to monomeric avidin beads. Bound peptides were eluted with 10% acetic acid and analyzed in the QTRAP mass spectrometer by LC/MS/MS. A search of the enhanced product ion spectra has revealed several FP-biotinylated peptides. One of these has been sequenced to date. This has resulted in the identification of Tyrosine 238 as the OP binding site of human transferrin. The sequence of the OP-peptide is KPVDEY*K where OP is bound to Y.

Tyr 172 of apolipoprotein. Peptide THLAPY*SDELR was found in the FP-biotinylated human plasma preparation. The FP-biotin was on Tyrosine 172 in accession # gi:178775 for human apolipoprotein. The MS/MS spectrum for parent 1873.9 contained 6 b-ions as well as a complete y-ion series y1-y11, and the FP-biotin ions at 227, 312, and 329. The data were convincing that we had correctly identified Tyr 172 of apolipoprotein as the OP-binding residue.

UNMC and Wistar. Proteins in a set of 6 gel slices were analyzed at UNMC on our QTRAP mass spectrometer and at Wistar on their LTQ-Thermo Finnigan mass spectrometer. The gel slices were from the same FP-biotinylated plasma preparation run on the same polyacrylamide gel. The results from both institutes were essentially the same, thus reassuring us that our techniques and equipment were satisfactory.

Table 1.1.3. FP-biotinylated peptides too short to assign to a particular protein.

sequence	labeled residue
YLK or YIK	Y
YTGK	Y
YPR	Y
YGPR	Y
YK	Y

Short peptides. A search of LC/MS/MS results for tryptic digests of FP-biotinylated peptides in human plasma allowed identification of the 5 peptides listed in Table 1.1.3. The OP is bound to tyrosine in each case. Leu and Ile have the same mass, making it impossible to distinguish between these amino acids in a mass spectrometer so that the first peptide in Table 1.1.3 could be either YLK or YIK. We have complete confidence in the peptide sequences and in the conclusion that FP-biotin is covalently attached to tyrosine. However, the peptides are too short to assign to a particular protein.

Discussion

Serine hydrolases are not the only OP binding proteins in human plasma. Our expectation when we started this project was that OP-labeled proteins would all be serine esterases and serine proteases. We expected butyrylcholinesterase to be the dominant OP-binding protein in human plasma (Fidder et al., 2002; Van Der Schans et al., 2004). Our results show that this expectation was not met. We have found that OP bind not only to serine esterases and serine proteases, but also to proteins that have an activated tyrosine. We have conclusively demonstrated that albumin, transferrin, and apolipoprotein bind OP on tyrosine. We expect to identify many more proteins that bind OP on tyrosine.

Our hypothesis is that each OP binds to a set of proteins, and that the sets do not overlap completely. For example, almost all OP bind to acetylcholinesterase and butyrylcholinesterase, but not all OP bind to albumin. We have been unable to demonstrate binding of VX or iso-OMPA to albumin. The identity of the proteins bound by a particular OP depends on the affinity of that particular OP, and therefore on the precise chemical structure of that OP. The OP literature has mostly studied nerve agents and DFP, thus limiting the view of the type of proteins capable of binding OP.

Motif for OP binding to tyrosine. We have positively identified Tyrosine 411 of albumin, Tyrosine 238 of transferrin, and Tyrosine 172 of apolipoprotein as sites that covalently bind OP in human plasma. A nearby arginine or lysine is likely involved in activating the tyrosine, lowering its pKa value and allowing the hydroxyl group to ionize at physiologic pH. We propose that a common motif for OP-reactive tyrosines may be the presence of a nearby arginine or lysine.

Relevance. An understanding of the set of proteins modified by exposure to a particular OP is expected to help in understanding why some people become chronically ill from a low dose exposure that has no effect on the average individual. A sensitive person may have a mutation in an OP-binding protein that reduces the person's tolerance to a low dose.

Knowledge of the set of proteins modified by OP exposure is also going to be useful for diagnosis of OP exposure. Proteins that have a longer half-life in the circulation can be tested weeks after exposure. Mass spectrometry assays rely on the ability of peptides to ionize in the electrospray; some OP-modified peptides may ionize more readily than others. Confirmation of exposure may be more certain if a set of proteins is proven to have been modified rather than a single protein.

The results of our work are expected to lead to an antibody based assay for OP exposure. The OP binding site in albumin is located on the surface of the albumin molecule, where the OP and the surrounding amino acids make an excellent epitope. A dipstick type assay is envisioned using antibody to OP-albumin.

TASK 1

Task 1. Identify proteins labeled by FP-biotin
1.1. Identify FP-biotin labeled proteins in human blood
1.2. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated human protein.

Relation to statement of work. Additional work on tasks 1.1 and 1.2 is reported.

Five tyrosines and one serine in human albumin are labeled by FP-biotin

Summary

We have previously identified Tyrosine 411 of human albumin as the binding site for FP-biotin, DFP, chlorpyrifos oxon, soman, sarin, and dichlorvos. The OP-labeled Tyr 411 peptide, but no other OP-labeled peptide, was released by digesting albumin with pepsin. In this work we tested the possibility that other residues in albumin were also modified by OP, and that they could be identified by using a different strategy to prepare peptides for mass spectrometry. We treated human plasma with FP-biotin, isolated an albumin containing fraction on an antibody column, reduced and alkylated the disulfides, digested with trypsin, and purified FP-biotinylated peptides by binding to monomeric avidin beads. A 5 µg aliquot of peptides was analyzed by LC/MS/MS on two different mass spectrometers: the QSTAR Elite and the QTRAP 2000. A total of six residues were found to covalently bind FP-biotin: tyrosine 138, 148, 401, 411, 452 and serine 232 of albumin. The reactivity of these residues can be explained by a nearby Arg or Lys that lowers the pKa of the hydroxyl group on Tyr and Ser. This information can be used to develop antibodies to diagnose OP exposure and to design peptide scavengers to treat OP toxicity.

Introduction

A 1975 publication by Means and Bender reported that one site in human albumin was rapidly labeled by p-nitrophenyl acetate, but that up to 5.2 sites were labeled when albumin was treated with a 9-fold excess of p-nitrophenyl ^{14}C-acetate (Means and Bender, 1975). Means and Bender did not identify the labeled residues. In 2007 we used mass spectrometry to demonstrate that the site rapidly labeled by p-nitrophenyl acetate was Tyr 411 (Masson et al., 2007). The same Tyr 411 residue is also rapidly labeled by soman, sarin, FP-biotin,

DFP, dichlorvos, and chlorpyrifos oxon ((Li et al., 2007). The parallel between the reactivity of albumin with p-nitrophenyl acetate and with OP led us to suspect that additional sites in albumin might react with OP. The goal of the present work was to determine if sites in addition to Tyr 411 could make a covalent bond with FP-biotin, and to identify the labeled residues.

Methods

Materials. FP-biotin was custom synthesized in the laboratory of Dr. Charles Thompson at the University of Montana, Missoula, MT. FP-biotin was dissolved in methanol and stored at -80˚C. Chlorpyrifos oxon was dissolved in ethanol and stored and -80˚C. Proteome Partitioning Kit, ProteomeLab IgY-12 High Capacity in Spin Column format contains IgY antibodies directed against the 12 most abundant proteins in human plasma (Beckman Coulter #A24331 S0510903). Porcine trypsin (Promega, Madison, WI; V5113 sequencing grade modified trypsin) at a concentration of 0.4 µg/µl in 50 mM acetic acid was stored at -80˚C. Pepsin (Sigma, St. Louis, MO; P6887 from porcine gastric mucosa) was dissolved in 10 mM HCl to make a 1 mg/ml solution, and stored at -80˚C. Monomeric avidin beads (#20228) were from Pierce Co. Fatty acid free human albumin Fluka 05418 was from Sigma/Aldrich.

Separation of Low and high abundance proteins in human plasma. 200 µl of human plasma (EDTA anticoagulant) were fractionated into low and high abundance proteins by processing 20 µl of plasma at a time on the Beckman Coulter Proteome IgY Spin column depletion kit. The yield of high abundance proteins was 400 µl with a protein concentration of 12 µg/µl. Of this, 123 µl was labeled with FP-biotin, 123 µl was used as a negative control, and the remainder was used for determination of protein concentration in a Coomassie blue assay.

High abundance proteins labeled with FP-biotin. The high abundance fraction of plasma has albumin protein as its major component. A 123 µl aliquot of the high abundance fraction was treated with 1.25 µl of 20 mM FP-biotin for 48 h at 37˚C. The final FP-biotin concentration was 200 µM. Excess FP-biotin was removed by dialysis against 2 x 4 liters of 10 mM ammonium bicarbonate.

Preparation of tryptic peptides. Proteins in 8 M urea were reduced with 5 mM dithiothreitol and alkylated with 40 mM iodoacetamide. The samples were diluted to reduce the concentration of urea to 2 M. Proteins were digested with a 1:50 ratio of protein to trypsin at 37˚C overnight. The trypsin was inactivated by heating the sample in a boiling water bath for 10 min. It was necessary to inactivate trypsin because trypsin could have destroyed the avidin protein used in the next step. FP-biotinylated peptides were purified by binding to 0.5 ml of monomeric avidin beads. Nonspecifically bound peptides were washed off with high salt buffers. The column was washed with water to remove salts, and FP-

biotinylated peptides were eluted with 10% acetic acid. The eluate was dried in a vacuum centrifuge in preparation for mass spectrometry.

The negative control was human plasma treated with everything except FP-biotin.

Mass spectrometry on QSTAR elite and QTRAP 2000. FP-biotinylated human plasma samples were sent to Applied Biosystems for analysis on their QSTAR elite LC/MS/MS system. They received 5 µg of the high abundance FP-biotinylated peptides purified from monomeric avidin beads. Data were analyzed with ProteinPilot 2.0 software.

A second 5 µg aliquot from the same protein preparation, and a negative control sample, were analyzed by LC/MS/MS on the QTRAP 2000 mass spectrometer (Applied Biosystems) at the University of Nebraska Medical Center. Data were analyzed with Analyst 1.4.1 software.

Pure albumin labeled with OP, digested with trypsin or pepsin. A 1 mg/ml solution of fatty acid free human albumin in 10 mM ammonium bicarbonate, 0.01% sodium azide, pH 8.3 was treated with a 15-fold molar excess of chlorpyrifos oxon or FP-biotin at room temperature overnight. A portion of the labeled albumin was reduced with 10 mM dithiothreitol, alkylated with 100 mM iodoacetamide, dialyzed against 2 x 4 liters of 10 mM ammonium bicarbonate, and digested with trypsin at a ratio of 1:30 for 10 h at 37˚C. Another aliquot of the carbamidomethylated OP-labeled albumin was digested with pepsin.

A 10 µl portion of FP-biotin or CPO labeled-albumin (not reduced and alkylated) was mixed with 10 µl of 1% TFA and digested with 2 µl of 1 mg/ml pepsin for 4 h at 37˚C. The digests were analyzed on a MALDI-TOF-TOF 4800 (Applied Biosystems) by spotting 0.5 µl with CHCA matrix.

Results and Discussion

Five tyrosines and one serine in human albumin were labeled with FP-biotin (Table 1.2.1). They are Tyr 138, 148, 401, 411, 452 and Ser 232. By convention residue numbers are for the mature protein, that is, minus the signal peptide, which for human albumin is -24.

Table 1.2.1. FP-biotinylated human albumin peptides identified by 2 mass spectrometers

sequence	labeled residue	residue number gi:28592	residue -24	QSTAR	QTRAP
Y*LYEIAR	Tyr 1	Tyr 162	138	yes	yes
KY*LYEIAR	Tyr 2	Tyr 162	138	yes	
HPY*FYAPEL	Tyr 3	Tyr 172	148	yes	
HPY*FYAPELL	Tyr 3	Tyr 172	148	yes	
HPY*FYAPELLF	Tyr 3	Tyr 172	148	yes	
HPY*FYAPELLFFAK	Tyr 3	Tyr 172	148	yes	yes
HPY*FYAPELLFFAKR	Tyr 3	Tyr 172	148	yes	
PY*FYAPELL	Tyr 2	Tyr 172	148	yes	
RHPY*FY	Tyr 4	Tyr 172	148	yes	
RHPY*FYAPEL	Tyr 4	Tyr 172	148	yes	
RHPY*FYAPELL	Tyr 4	Tyr 172	148	yes	
RHPY*FYAPELLF	Tyr 4	Tyr 172	148	yes	
RHPY*FYAPELLFF	Tyr 4	Tyr 172	148	yes	
RHPY*FYAPELLFFAK	Tyr 4	Tyr 172	148	yes	yes
RHPY*FYAPELLFFAKR	Tyr 4	Tyr 172	148	yes	
QNCELFKQLGEY*K	Tyr 12	Tyr 425	401	yes	yes
Y*TK	Tyr 1	Tyr 435	411	no	yes
MPCAEDY*LSVVLNQLCVLHEK	Tyr 7	Tyr 476	452	yes	yes
RMPCAEDY*LSVVLNQLCVLHEK	Tyr 8	Tyr 476	452	yes	
AEFAEVS*K	Ser 7	Ser 256	232	yes	no

Residue numbering for accession # gi:28592 begins with the translation initiation Methionine. The signal peptide contains 24 amino acids. The mature albumin residue numbers are smaller by 24.

Supporting MS/MS spectra for all but the Tyr 411 assignments are in Figures 1.2.1, 1.2.2, 1.2.3 and 1.2.4 for data obtained by the QSTAR Elite mass spectrometer. Peptide Y*TK containing Tyr 411 was not found in the QSTAR analysis because peptides smaller than 5 residues were not accepted for analysis by the search engine. However, the Y*TK peptide was found by manual analysis (Figure 1.2.5) of data from the QTRAP 2000 mass spectrometer.

The residue we have previously identified as modified by FP-biotin is Tyr 411 in the sequence YTK (Li et al., 2007). Tyr 411 is responsible for the esterase activity of albumin toward p-nitrophenyl acetate (Means and Wu, 1979; Masson et al., 2007).

No FP-biotinylated peptides were found in the control sample that had not been treated with FP-biotin.

Figure 1.2.1. Human albumin peptide Y*LYEIAR labeled with FP-biotin on Tyr 138, analyzed on the QSTAR Elite LC/MS/MS system.

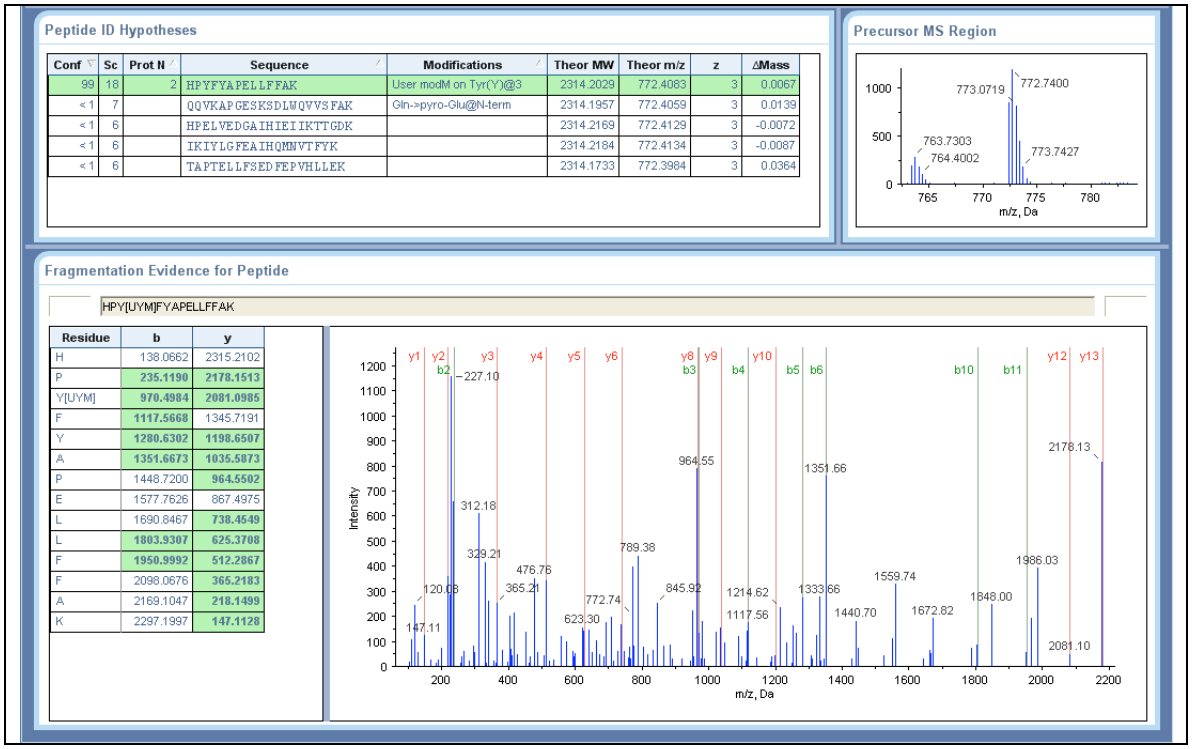

Figure 1.2.2. Human albumin peptide HPY*FYAPELLFFAK labeled with FP-biotin on Tyr 148.

Figure 1.2.3. Human albumin peptide QNCELFEQLGEY*K labeled with FP-biotin on Tyr 401.

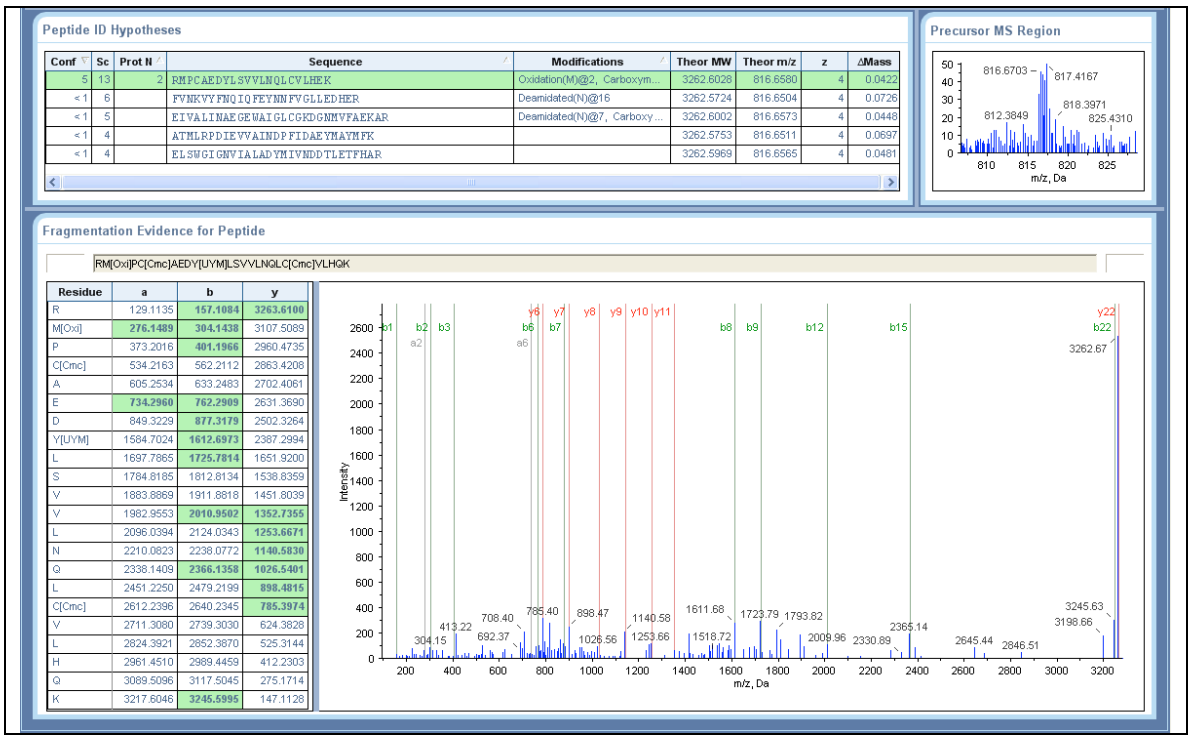

Figure 1.2.4. Human albumin peptide RMPCAEDY*LSVVLNQLCVLHEK labeled with FP-biotin on Tyr 452, analyzed on the QSTAR Elite mass spectrometer.

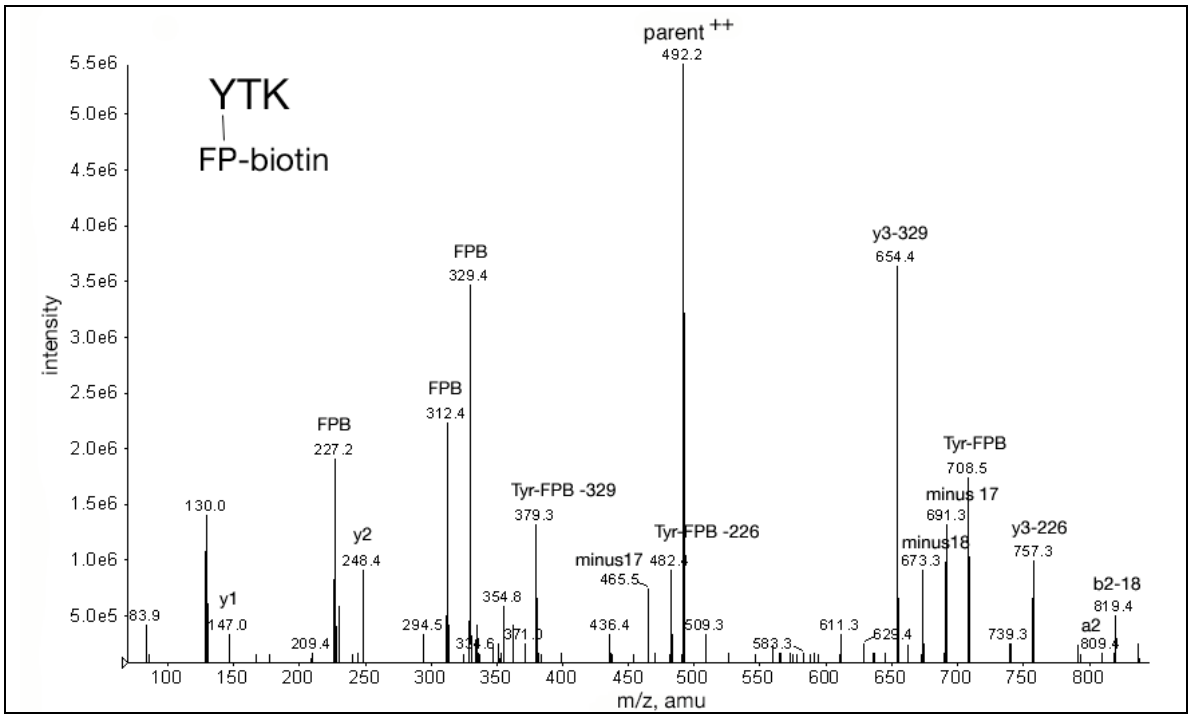

Figure 1.2.5. Human albumin peptide YTK labeled with FP-biotin on Tyrosine 411. The characteristic fragment ions of FP-biotin at 227, 312, and 329 are present. The doubly charged parent ion (492.2) has a singly charged mass of 983.5. The MS/MS spectrum was obtained on the QTRAP 2000 mass spectrometer.

Evaluation of data in Figures 1.2.1-1.2.5 for labeling of tyrosine. The characteristic ions resulting from fragmentation of FP-biotin are present at 227, 312, and 329 amu, supporting the conclusion that the peptides in Figures 1.2.1-1.2.5 are labeled with FP-biotin. None of the spectra have a 591 ion, thus eliminating the possibility of labeling on serine (Schopfer et al., 2005). The b-ion and y-ion masses support the conclusion that the FP-biotin label is on tyrosine. The spectra have continuous y-ion and b-ion series, a result that supports the amino acid sequence assigned. All major peaks were assigned either by the software or by manual inspection. Ions not recognized by the software were found by manual inspection to be doubly charged ions, or singly charged ions minus 18 for loss of water, or singly charged ions minus FP-biotin fragments of 227, 312, or 329 amu. It is concluded that the MS/MS data in Figures 1.2.1-1.2.5 provide convincing proof that albumin is covalently labeled by FP-biotin on tyrosines 138, 148, 401, 411, and 452.

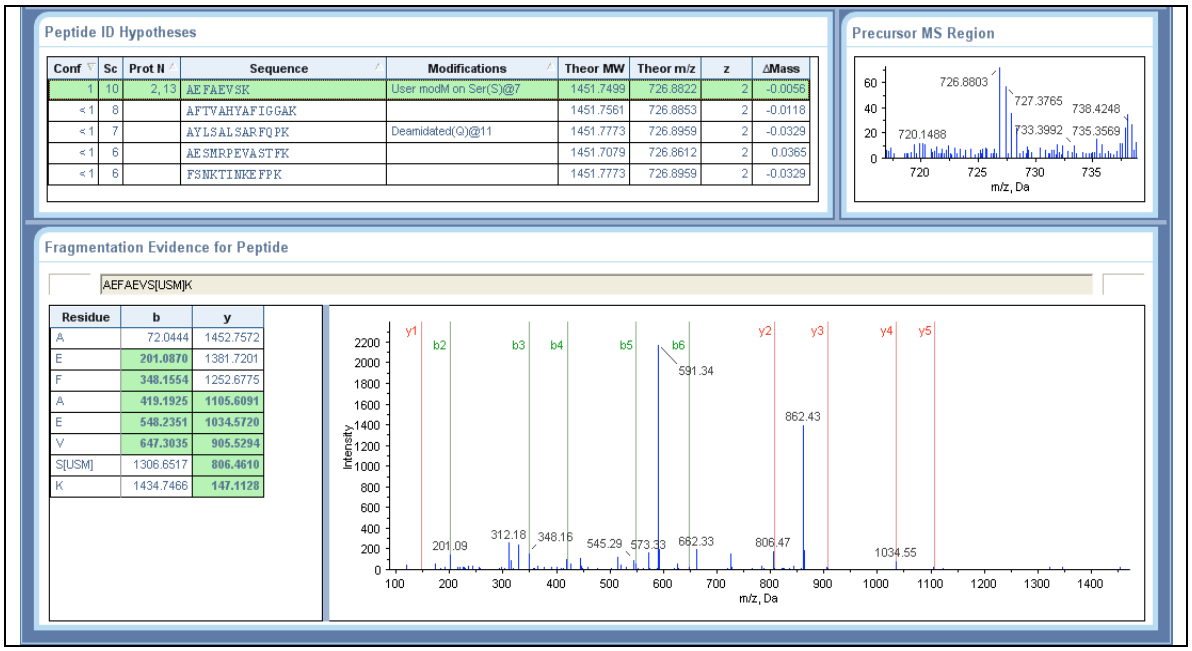

Figure 1.2.6. Human albumin peptide AETAEVS*K labeled with FP-biotin on Ser 232.

Evaluation of data in Figure 1.2.6 for labeling of serine. Figure 1.2.6 indicates singly charged y-ions (y1-y5) and singly charged b-ions (b2-b6), but leaves several major peaks unassigned. Our experience with fragmentation of OP-labeled serine allows us to assign these additional peaks. The intense peak at 591.34 is intact FP-biotin minus the fluoride ion (Schopfer et al., 2005). Peaks at 227, 312, and 329 are fragments of FP-biotin. The intense peak at 862.43 is y8*, that is, the dehydroalanine form of the 8-residue peptide from which the entire FP-biotin and a water molecule have been lost, leaving behind dehydroalanine in place of serine. Other dehydroalanine ions are also present: y3* at 315.21, y4* at 444.25, y5* at 515.28, and y6* at 662.34. It is noteworthy that only a portion of the FP-biotin labeled serine fragmented to dehydroalanine. Another portion retained the FP-biotin label to yield y-ions and b-ions with an added mass of +572. The software correctly identified modified peptides containing an added mass of +572. This analysis explains all major peaks in the MS/MS spectrum and therefore provides convincing proof that Ser 232 of human albumin is labeled with FP-biotin.

The FP-biotinylated Ser 232 peptide of albumin was not found by the QTRAP 2000 mass spectrometer, suggesting that this peptide was present in very low amounts, detectable only by the more sensitive QSTAR Elite mass spectrometer.

The QSTAR Elite mass spectrometer outperforms the QTRAP 2000. The same plasma samples, containing the same amount of protein (5 micrograms), were analyzed on the QTRAP 2000 and QSTAR elite mass spectrometers. The QTRAP 2000 is equipment on the University of Nebraska

campus. The QSTAR Elite mass spectrometer is at Applied Biosystems in Framingham, MA where James E Carlson, an LC/MS product applications specialist, processed our sample. The QSTAR Elite identified many more OP-albumin peptides than the QTRAP 2000.

However, the QSTAR Elite mass spectrometer did not identify the FP-biotinylated YTK peptide. The Mascot search engine is limited to peptides containing at least 6 residues because peptides with fewer than 6 residues cannot be assigned to a particular protein with confidence. This explains why a Mascot search did not find the 3-residue FP-biotin labeled YTK peptide. We found the labeled YTK peptide by manually searching the MS/MS spectra for the presence of the characteristic fragmentation ions of FP-biotin at 227, 312 and 329 amu, using the extracted ion feature of the Analyst 1.4.1 software. Then we analyzed the fragment ion masses to obtain the amino acid sequence.

Location of OP-labeled residues on the surface of the albumin molecule. The crystal structure of human albumin shows that the OP-labeled residues are exposed to the solvent where they can react with OP (Figure 1.2.7). Human albumin (accession # gi:28592) has 18 Tyrosines and 24 Serines, but only 5 Tyrosines and 1 Serine made a covalent bond with FP-biotin. Their special reactivity may be explained by a nearby Arginine or Lysine that stabilizes the ionized hydroxyl of Tyrosine or Serine.

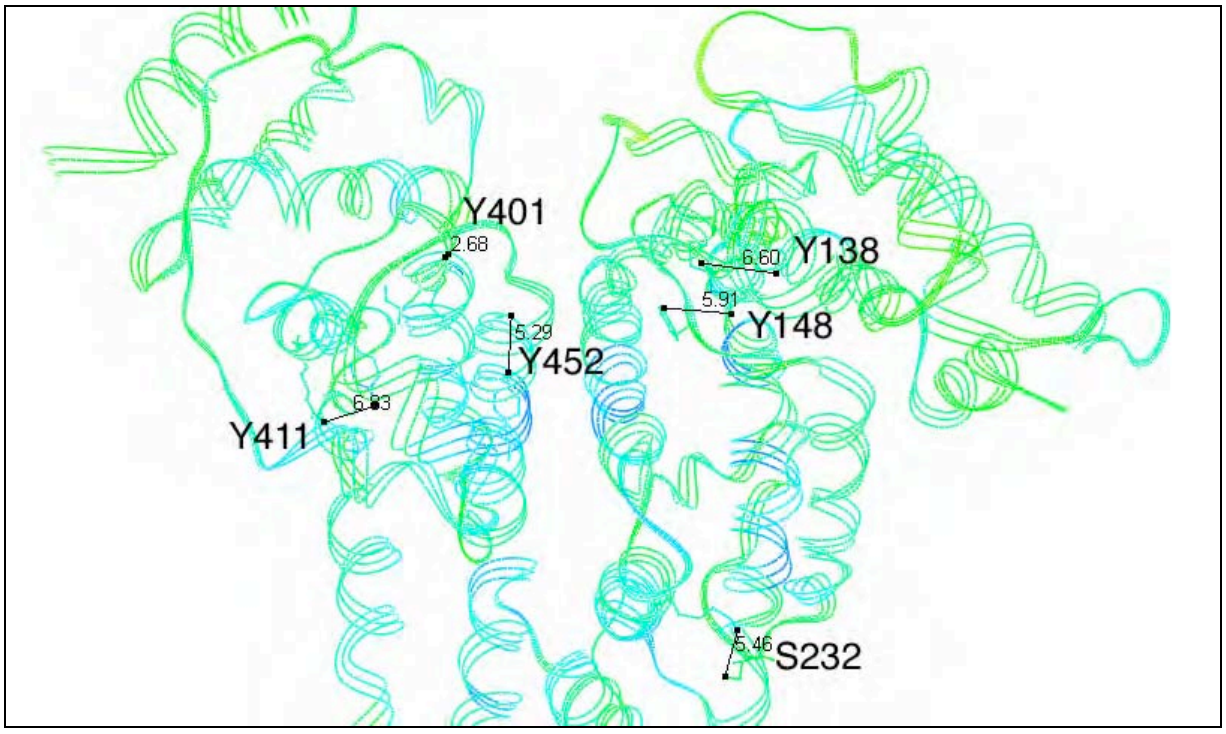

Figure 1.2.7. Crystal structure of human albumin (PDB code 1uor). Distances between OP-reactive Tyr or Ser and the nearest Arg or Lys are indicated.

The distances between OP-reactive side chains and the nearest Arg or Lys were measured in human albumin crystal structures deposited in the Protein Data Bank. The measured distances ranged from 2.68 to 8.16 Angstroms, and depended on the crystal structure examined (Table 1.2.2). The fact that distances were different in each crystal structure suggests that residue positions were flexible, an indication of solvent accessibility. The Arg or Lys presumed to activate the Tyr was adjacent to Tyr in two examples. It might be possible to design a small peptide that has the ability to scavenge OP.

Table 1.2.2. Distance between OP-reactive amino acids and the nearest Arg or Lys

	sequence	PDB 1uor	PDB 1bm0
Tyr 138	LKKY*LYE	6.60 to Lys 137	7.98 to Arg 160 15.3 to Lys 137
Tyr 148	HPY*FYAP	5.91 to Lys 106	5.27 to Lys 106
Tyr 401	LGEY*KFQ	2.68 to Arg 428	4.84 to Lys 521 7.74 to Arg 428
Tyr 411	LVRY*TKK	6.83 to Arg 410	4.53 to Arg 410
Tyr 452	CAEDY*LS	5.29 to Lys 432	4.47 to Lys 436 5.69 to Lys 432
Ser 232	AEVS*KLV	5.46 to Lys 212	8.16 to Lys 212

Distance is in Angstroms for albumin crystal structures in Protein Data Bank accession codes 1uor and 1bm0.

Relative reactivity. The MS/MS data in Figures 1.2.1-1.2.6 provide no information regarding the extent of labeling on each tyrosine because only the labeled peptides and not the corresponding unlabeled peptides were in the sample. Therefore albumin samples containing both the unlabeled and labeled peptides were prepared and spotted on a MALDI target plate. The labeled albumin was reduced, alkylated, and digested with trypsin. MS and MS/MS spectra identified two residues in albumin labeled with FP-biotin; they were Tyr 411 in Y*TK and Tyr 138 in Y*LYEIAR. By comparing the cluster areas for the unlabeled and labeled peaks, we calculated that 99% of the Tyr 411 and 70% of the Tyr 138 carried the FP-biotin label. No other FP-biotinylated albumin peptides were found by MALDI-TOF, even though the albumin disulfides had been reduced and alkylated. In a second labeling experiment, chlorpyrifos oxon labeled albumin was reduced, alkylated, digested with trypsin, and analyzed by MALDI-TOF. It was found that 85% of Tyr 411 sites and 83% of Tyr 138 sites were labeled with CPO.

Only two rather than 6 residues in albumin were found to be labeled in the experiments with pure albumin analyzed by MALDI-TOF. This could be explained by ion suppression or to low reactivity of other sites.

The experiments of Means and Bender with p-nitrophenyl acetate (Means and Bender, 1975), and of Means and Wu with DFP and p-nitrophenyl acetate (Means and Wu, 1979) show that one site in albumin is more reactive than

others, and that this same site is labeled by both p-nitrophenyl acetate and DFP. Means concluded that the rapidly reacting site is Tyr 411. We agree that Tyr 411 reacts rapidly with both p-nitrophenyl acetate and OP, but we have not yet excluded the possibility that Tyr 138 also reacts rapidly.

Significance.

OP-labeled albumin could be used to make antibodies for detection of OP exposure. The surface location of the OP-binding sites in albumin means the epitopes are available for reaction with antibody, unlike the situation for acetylcholinesterase and butyrylcholinesterase where the OP binding site is buried deep within the molecule making it unavailable to antibodies.

Identification of an OP binding motif that is different from the well defined active site serine consensus sequence GXSXG, may lead to the design of a short peptide that will serve as a bioscavenger of OP.

Task 1

Task 1. Identify proteins labeled by FP-biotin
1.3. Identify FP-biotin labeled proteins in mouse blood
1.4. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated mouse protein.

Relation to statement of work. Results for tasks 1.3 and 1.4 are reported.

Tasks 1.3 and 1.4

OP-labeled proteins in mouse plasma

Summary
The goal was to identify proteins in mouse plasma that bind FP-biotin. Mass spectrometry identified three carboxylesterases in mouse plasma, the major carboxylesterase being ES1. Additional FP-biotinylated proteins were albumin, two forms of hemoglobin, serine protease inhibitor, contraspin, and plakoglobin.

Introduction

The reason for duplicating our efforts in mouse and human plasma is that the mouse will be used for in vivo studies. Once we know the proteins to look for, we will inject mice with various doses of FP-biotin and quantify the amount of each labeled protein by mass spectrometry. This will indicate which proteins are likely to be important in FP-biotin toxicity.

It is known that mouse plasma differs from human plasma in that mouse plasma is rich in carboxylesterase but human plasma has no carboxylesterase (Maxwell et al., 1987; Li et al., 2005).

Materials and Methods

The materials and methods are the same as described for Tasks 1.1 and 1.2. An 80 µl aliquot of mouse plasma from strain 129Sv mice was depleted of albumin by chromatography on Affi-gel blue, and labeled with FP-biotin. The FP-biotinylated proteins were purified by binding to immobilized avidin beads. The proteins were released from the beads by boiling in SDS gel loading buffer, and separated on an SDS gel. Coomassie stained bands were cut out. Each band was treated with dithiothreitol to reduce protein disulfide bonds, followed by

alkylation with iodoacetamide. Proteins were digested with trypsin and the tryptic peptides were extracted from the gel. The peptides were subjected to LC/MS/MS analysis on the QTRAP 2000 mass spectrometer. Proteins were identified by analysis of fragmentation patterns using Mascot software.

Results and Discussion

Table 1.3.1 lists the FP-biotinylated proteins identified in mouse plasma.

Table 1.3.1 FP-biotin binding proteins in mouse plasma (strain 129Sv).

protein	MOWSE	# peptides	MW	Gi #	OP binding site
ES1 carboxylesterase	845	18	61266	22135640	GESSG
carboxylesterase precursor	782	16	61140	2921308	GESSG
liver carboxylesterase N precursor, lung surfactant convertase isoform 3	508	10	60782	82918153	GSSAG
albumin	506	10	68693	29612571	RYTQK
hemoglobin alpha	157	3	15085	49900	-
hemoglobin beta	130	3	15840	1183932	-
serine protease inhibitor	117	2	46866	15079234	-
contraspin	97	3	46673	54173	-
contraspin	96	2	46965	50442	-
plakoglobin	37	2	68151	423532	-

MOWSE score is the probability of a match between the experimental data and the peptide mass in the database. MOWSE scores greater than 69 are significant ($p<0.05$). # peptides is the number of peptides whose sequence matched the protein. MW is the molecular weight. Gi# is the accession number. The active site serine for OP binding is S in the sequence GXSXG. The active site tyrosine for OP binding in albumin is Y.

Carboxylesterases. Mouse plasma was analyzed on the QTRAP three times. Three carboxylesterases were identified. The major carboxylesterase was ES1. A good score for ES1 was found in all 3 experiments. The carboxylesterase precursor was found in 2 experiments, while the liver carboxylesterase was found only in one experiment, after the sample had been concentrated prior to analysis on the mass spectrometer. This suggests that the carboxylesterase precursor and the liver carboxylesterase have a lower abundance than ES1 in mouse plasma.

In a previous mass spectrometry study we also found ES1, but no other carboxylesterase, suggesting that ES1 is indeed the most abundant carboxylesterase in mouse plasma (Peeples et al., 2005). Others have also found ES1 (gi: 22135640) in mouse plasma by LC/MS/MS (Bhat et al., 2005). Biochemical characterization of the carboxylesterases in mouse serum classified ES1 as the principal carboxylesterase in mouse serum (Otto et al., 1981).

The question of which carboxylesterases are present in mouse plasma is relevant to the effort to make a carboxylesterase knockout mouse. Mice have 16

carboxylesterase genes located in two clusters on chromosome 8 (Mus musculus chromosome 8, reference assembly; NC 000074). From our mass spectrometry results it can be expected that knockout of carboxylesterase ES1 will substantially reduce the carboxylesterase activity of mouse blood but will not eliminate it completely.

Albumin. Mouse plasma was depleted of albumin prior to labeling and mass analysis, because protein blots had revealed that albumin bound most of the FP-biotin. Albumin scavenged 1000 more molecules of FP-biotin than were scavenged by butyrylcholinesterase (Peeples et al., 2005) in a living mouse. Despite the depletion step, there was enough albumin left in the 2.4 mg of processed protein to show up as an OP-labeled tryptic peptide in the mass spectrometer. The mouse albumin peptide that binds OP has the sequence RYTQK with FP-biotin bound to Y.

Other OP-labeled proteins. We are not convinced that hemoglobin, contraspin, and plakoglobin are labeled with OP. These proteins could have bound to avidin beads nonspecifically. We will require additional proof before we have a conclusive answer. The additional proof is of two types. 1) Protein blots of purified proteins treated with FP-biotin must hybridize with Streptavidin Alexafluor 680. This type of experiment conclusively proves that a protein covalently binds FP-biotin, but it does not identify the site of binding. 2) The peptide containing the FP-biotin label must ionize in the mass spectrometer and must fragment to allow its amino acid sequence to be deduced from the fragmentation pattern. Evidence of this type provides irrefutable proof.

Significance. The OP-binding proteins in mouse plasma are expected to parallel the OP-binding proteins in human plasma, with the major exception of carboxylesterase. Human plasma contains no carboxylesterase, whereas mouse plasma is rich in carboxylesterase (Li et al., 2005). It is important to know the identity of the OP-binding proteins in mouse plasma because the mouse will be used for in vivo studies. In the future we will inject mice with various OP and use mass spectrometry to identify and quantify the proteins that have been modified by each OP.

Task 1

1.5. Inject mice with FP-biotin and identify the FP-biotinylated proteins in mouse blood.
reported July 20, 2007

Relation to statement of work. Results for task 1.5 are reported.

Task 1.5

FP-biotinylated proteins in mouse blood after in vivo treatment with FP-biotin

Summary

A mouse was injected intraperitoneally with 5 mg/kg FP-biotin, a dose that caused no signs of toxicity, and that did not inhibit acetylcholinesterase activity in blood. Butyrylcholinesterase in plasma was inhibited 37%. FP-biotinylated proteins in mouse plasma were purified by binding to avidin beads. The beads were washed with 0.2% SDS to remove nonspecifically bound proteins and loaded on an SDS polyacrylamide gel. Coomassie stained bands were excised from the gel. Proteins were reduced, alkylated with iodoacetamide, and digested with trypsin. The digest from each gel band was analyzed by LC/MS/MS. Mass spectrometry identified FP-biotin labeled albumin (accession # gi5915682) and ES1 carboxylesterase (accession # gi22135640). Two additional methods to identify FP-biotin labeled proteins in mouse blood were used: 1) gel electrophoresis followed by transfer to a membrane and hybridization with a fluorescent probe, and 2) enzyme activity assays. For these assays mice were treated intraperitoneally with 18.8 mg/kg FP-biotin. This dose inhibited acetylcholinesterase 56%, butyrylcholinesterase 82% and carboxylesterase 81%. These additional methods identified acetylcholinesterase, butyrylcholinesterase, and carboxylesterase in mouse blood as proteins that bind FP-biotin.

A publication describing these results is attached.
Peeples ES, Schopfer LM, Duysen EG, Spaulding R, Voelker T, Thompson CM, Lockridge O.
Albumin, a new biomarker of organophosphorus toxicant exposure, identified by mass spectrometry.
Toxicol Sci. 2005 Feb;83(2):303-12.

TASK 2

Task 2. Identify proteins labeled by DFP
2.1. Label pure human butyrylcholinesterase with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation.
report for 20 Jan 2007 sent 7 March 2007

Relation to statement of work. Results for task 2.1 are reported.

The active site serine of DFP-labeled human butyrylcholinesterase loses DFP and water in the collision chamber of the mass spectrometer to yield dehydroalanine

Summary

Our long range goal is to develop a sensitive method for diagnosing OP exposure. For this purpose we plan to use a multiple reaction monitoring method on the mass spectrometer. The method requires knowledge of how OP-modified proteins fragment in the mass spectrometer. We chose to examine human butyrylcholinesterase (BChE) modified with OP because BChE is highly reactive with a variety of OP, and BChE is present in serum. These features make BChE a useful biomarker of exposure. Human BChE reacts stoichiometrically with OP to covalently modify serine 198 in the active site. We have already acquired MS/MS spectra for human BChE labeled with FP-biotin, soman, sarin and found that these OP break off in the collision chamber taking a molecule of water with them. In place of the active site serine, the peptide is left with dehydroalanine. We expected to find the same pattern with DFP-labeled human BChE, and our expectation was fulfilled. In addition to confirmation that dehydroalanine is produced with a variety of OP, our MS/MS data identify peaks that are consistently most intense. This information is valuable for developing a sensitive multiple reaction monitoring assay for diagnosis of OP exposure.

Introduction

Human BChE is highly reactive with OP. The active site serine of human BChE makes a stable covalent bond with OP that may persist for the lifetime of the OP-labeled BChE in the circulation. These features make BChE a useful biomarker of OP exposure. Knowledge of how an OP-labeled protein fragments in the mass spectrometer is necessary for the design of sensitive analytical procedures for detecting OP exposure.

Materials and Methods

Pure human BChE. BChE was purified from outdated human plasma by ion exchange chromatography at pH 4.0, affinity chromatography on procainamide-Sepharose, and HPLC on a Protein Pak DEAE Anion Exchange column (Lockridge et al., 2005). 1.28 mg of BChE (14.7 nanomoles) with an activity of 135.3 units/ml in a total of 6.8 ml were used for this experiment. This amount of BChE is present in 300 ml plasma. Pure BChE has a specific activity of 720 units/mg where protein concentration is calculated from absorbance at 280 nm. A 1 mg/ml solution of pure BChE has an absorbance of 1.8 at 280 nm.

BChE activity. Activity was measured with 1 mM butyrylthiocholine and 50 µM dithiobisnitrobenzoic acid in 0.1 M potassium phosphate pH 7.0 at 25˚C. Increase in absorbance at 412 nm was recorded for about 1 min in a Gilford spectrophotometer in a 2 ml reaction. The molar extinction coefficient for calculating product formed per min was 13,600 M^{-1} cm^{-1}. Units of activity are micromoles per min.

DFP. Diisopropylfluorophosphate (DFP) is a liquid with a concentration of 5.73 M (D-0879, Sigma, St. Louis, MO). A 0.3 M stock solution of DFP was prepared in ethanol and stored at -80˚C.

Reaction of DFP with BChE. 6.8 ml of BChE in pH 7 buffer was treated with 1 µl of 0.3 M DFP, a 20-fold molar excess of DFP over BChE. After 1 h at room temperature, the BChE activity was zero.

Trypsin digestion. The DFP-labeled BChE was dialyzed against 20 mM ammonium bicarbonate in an Amicon diaflo filtration unit with a PM10 membrane, and reduced in volume to 0.4 ml. The 0.4 ml of BChE was denatured by placing the tube in a boiling water bath for 10 min. The denatured BChE was digested with 10 µg of porcine trypsin (V5113C sequencing grade modified trypsin in 50 mM acetic acid, Promega) at 37˚C for 5.5 h.

HPLC to purify the active site peptide. The digest was injected onto a reverse phase Zorbax column 300SB C3 4.6 mm x 15 cm equilibrated with 0.1% TFA. Peptides were eluted with a gradient increasing to 60% acetonitrile in 60 min. 1

ml fractions were collected. A 1 µl aliquot from each 1 ml fraction was analyzed on the MALDI-TOF to identify fractions containing the expected aged and unaged active site peptides with masses of 3052.5 and 3094.3. The 3052.5 peak eluted in fractions 34-40. The 3094.3 peak eluted in fractions 38-40. Fractions 36 and 39 were selected for further analysis. Samples were dried under vacuum and dissolved in 100 µl of 50% acetonitrile, 0.1% formic acid.

Quadrupole mass spectrometer. MS/MS spectra were acquired on a QTRAP 4000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA) with a nano electrospray ionization source. DFP-labeled BChE digested with trypsin was infused into the mass spectrometer via a fused silica emitter (360 µm o.d., 20 µm i.d., 10 µm taper from New Objective, Woburn, MA), using a Harvard syringe pump to drive a 100 µl Hamilton syringe equipped with an inline 0.25 micron filter, at a flow rate of 0.35 µl/min. Positive ion spectra were obtained. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with a collision energy of 40-50 volts and a nitrogen gas pressure of 4×10^{-5} Torr. The final enhanced product ion spectra were the average of 200-250 scans.

Data analysis. The Protein Prospector website hosted by the University of California San Francisco was used for *in silico* digestion and calculation of theoretical monoisotopic peptide masses. The MS/MS fragment ion calculator on the Proteomics Toolkit website was used for predicting a, b, and y ion masses. MS/MS data were analyzed with Mascot (Matrix Science) software and Analyst 1.4.1 software from ABI.

Results

Parent ion masses. One residue in BChE is covalently modified by DFP and this residue is Serine 198. The Ser 198 tryptic peptide has the sequence SVTLFGE**S**AGAASVSLHLLSPGSHSLFTR. The masses of +1, +2, +3, and +4 charge states for the DFP modified peptide are listed in Table 2.1.1. The aged monoisopropylphosphate-labeled peptide has a singly charged mass of 3052.3. The unaged diisopropylphosphate-labeled peptide has a singly charged mass of 3094.3.

Table 2.1.1. Average masses of the aged (+122) and unaged (+164) DFP-labeled active site BChE tryptic peptide in various charge states. Parent ion masses for MS/MS fragmentation were chosen from this list.

sample	+1	+2	+3	+4
aged DFP-BChE 2930.3 + 122	3052.3	1526.6	1018.1	763.8
not aged DFP-BChE 2930.3 + 164	3094.3	1547.6	1032.1	774.3

MS/MS spectra. The infused sample was first scanned to determine which of the theoretical masses in Table 2.1.1 were present. We found 1018.3, 763.9, 1032.1 and 774.3. Peak intensities for the aged DFP labeled parent ion were higher than for the nonaged DFP parent ion. Each of the parent ions was fragmented in the collision chamber to produce MS/MS spectra. Figure 2.1.1 shows the MS/MS spectrum for the triply charged parent ion 1018.3. All major peaks are assigned. The series of y-ions and b-ions are completely consistent with the sequence of the active site peptide of BChE.

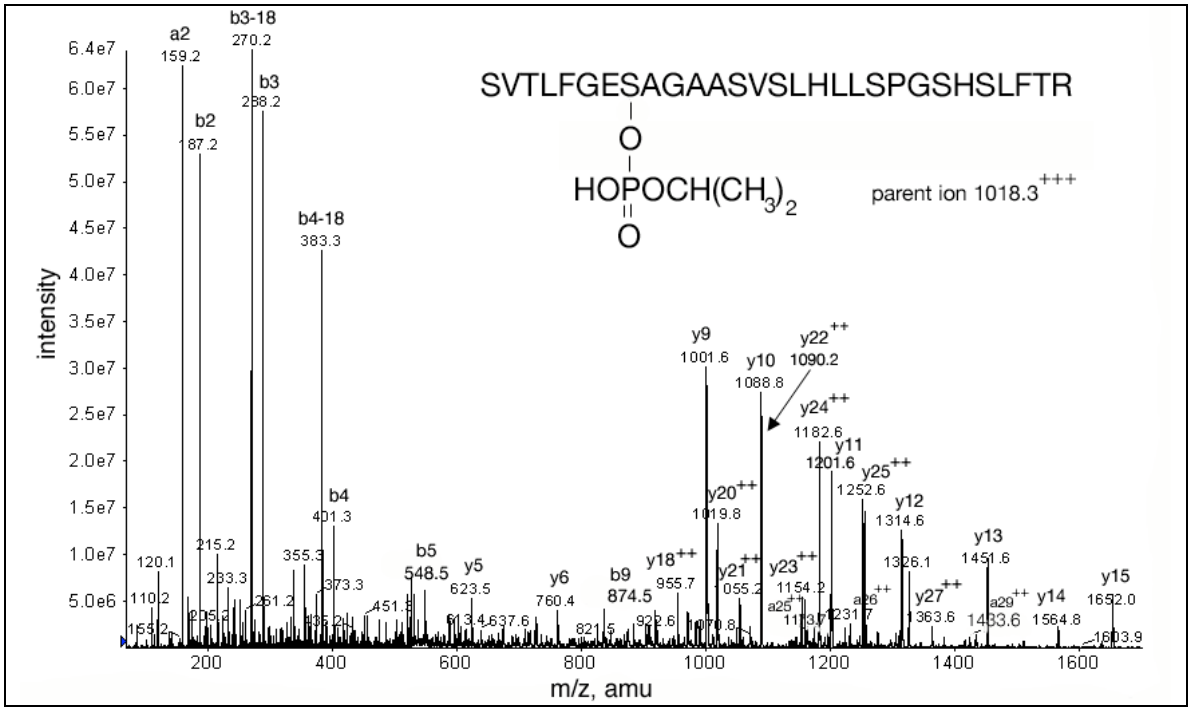

Figure 2.1.1. MS/MS spectrum of the aged DFP-labeled BChE active site tryptic peptide.

MS/MS spectra for parent ions 763.9, 1032.1 and 774.3 were almost identical to the spectrum for parent ion 1018.3 in Figure 2.1.1. The parent ions had different charges, and were modified by either aged or not aged DFP, but the MS/MS spectra were the same because the DFP was immediately released along with a water molecule when the ion entered the collision chamber.

The fragment ion masses for SVTLFGE**S**AGAASVSLHLLSPGSHSLFTR fit a scheme where all fragments that include **S** have a mass that is smaller by 18 amu than expected for serine. The active site serine has lost water and become dehydroalanine. The active site serine has also lost all atoms from DFP. None of the a-ions, b-ions, or y-ions include any portion of the DFP molecule. Only the parent ion includes DFP.

The fragment ion masses in Figure 2.1.1 that include dehydroalanine are listed in Table 2.1.2. One ion with a charge of +1 was found, the b9 ion at 874.5. Three a-ions and 5 y-ions with a charge of +2 are present in Figure 2.1.1. The

doubly charged y-ions are the most intense and therefore the most useful. The theoretical number of dehydroalanine ions is much greater than the 9 observed. Theoretically one could expect a8-a29, b8-b29, and y22-y29 in both charge states for a total of 104 ions to contain dehydroalanine. The nine dehydroalanine ions that were found are enough to convince that the peptide had been labeled with an OP. The mass of the parent ion identifies the OP.

Table 2.1.2. Dehydroalanine fragment ions in Figure 2.1.1.

+1	mass	+2	mass	+2	mass
b9	874.5	a25	1173.7	y22	1090.2
		a26	1231.7	y23	1154.2
		a29	1433.6	y24	1182.6
				y25	1256.3
				y27	1363.6

Discussion

Dehydroalanine. There is only one published MS/MS spectrum of an OP-labeled human BChE tryptic peptide (Fidder et al., 2002). The soman labeled BChE yielded a mixture of y-ions, some of which contained dehydroalanine in place of the active site serine and others that contained methylphosphonic acid attached to serine. We have also prepared MS/MS spectra of soman-BChE, sarin-BChE, and FP-biotin-BChE tryptic peptides (unpublished). We find that the most intense y-ions are dehydroalanine ions. Only very weak peaks can be interpreted as ions containing OP bound to serine. In our work with DFP we saw only dehydroalanine y-ions. The retention of OP-serine adducts during fragmentation may depend on the collision energy.

When Fidder produced peptides by digestion with pepsin, he saw only dehydroalanine ions when he fragmented soman-, sarin-, and paraoxon-labeled human BChE peptides (Fidder et al., 2002). None of the ions retained any part of the OP. Similarly, chymotryptic peptides of soman-, sarin-, and VX-labeled human BChE yielded only dehydroalanine ions and no ions that contained any part of the OP (Tsuge and Seto, 2006).

Conclusion

The general rule for OP-serine adducts is that the fragmentation process releases the entire OP and simultaneously releases a water molecule from the active site serine, converting the serine to dehydroalanine. Only in rare cases does the OP remain bound to serine during fragmentation.

The rule for fragmentation of OP-tyrosine adducts is very different. The phosphate always remains bound to tyrosine. The alkyl portions of the OP are released, but the phosphate or methylphosphate remains on tyrosine.

Knowledge of these fragmentation patterns is useful when using mass spectrometry to search for proteins modified by exposure to OP.

TASK 2

Task 2. Identify proteins labeled by DFP
2.2. Label pure human albumin with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation.

Relation to statement of work. Results for task 2.2 are reported.

Task 2.2

Human albumin is labeled on Tyrosine 411 (Tyr 435) by DFP

Summary

Our goal was to determine whether diisopropylfluorophosphate (DFP) covalently binds to human albumin. A second goal was to determine the fragmentation pattern of the DFP-albumin tryptic peptide. Human albumin was treated with DFP at alkaline pH, digested with pepsin at pH 2.3, and analyzed by MADLI-TOF mass spectrometry. Two singly charged peaks, 1718 and 1831 m/z, corresponding to the unlabeled peptide fragments containing the active site Tyr 411 residue, were detected in all samples. The sequences of the two peptides were VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL. The DFP adducts of these peptides had masses of 1881 and 1994; these masses fit a mechanism whereby DFP bound covalently to Tyr 411. The binding of DFP to Tyr 411 of human albumin was confirmed by electrospray tandem mass spectrometry and analysis of product ions. The fragmentation pattern yielded characteristic ions that will be useful for selected ion monitoring of DFP exposure. The OP-albumin adduct did not lose an alkoxy group, leading to the conclusion that aging did not occur. The presence of Tyr 411 on an exposed surface of albumin suggests that an antibody response could be generated against OP-albumin adducts.

Introduction

The acute toxicity of organophosphorus toxicants (OP) is known to be due to inhibition of acetylcholinesterase. However, other proteins also bind OP though

their role in toxicity is less defined (Casida and Quistad, 2004). Albumin is a potential new biomarker of OP exposure. Mice treated with a nontoxic dose of a biotinylated nerve agent analog, FP-biotin (10-fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide), had 1000 times more FP-biotinylated albumin than FP-biotinylated butyrylcholinesterase in their blood (Peeples et al., 2005).

Albumin has been shown to covalently bind radiolabeled diisopropylfluorophosphate (DFP). Human albumin incorporated 1 mole of DFP per mole of albumin when 20-70 μM albumin was incubated with a 7-fold molar excess of DFP at pH 8.0 for 2 h at 23˚C (Means and Wu, 1979; Hagag et al., 1983). Bovine albumin also incorporated 1 mole of DFP per mole of albumin (Murachi, 1963). The site of covalent binding of DFP to human albumin was identified by amino acid sequencing. The labeled peptide had the sequence ArgTyrThrLys with DFP bound to Tyr (Sanger, 1963). Later, when the complete amino acid sequence of human albumin was known, the active site tyrosine was identified as Tyr 411 (Tyr 435 when residue #1 is Met of the signal peptide). Mass spectrometry identified Tyr 410 of bovine albumin (equivalent to Tyr 411 of human albumin) as the covalent binding site for FP-biotin (Schopfer et al., 2005). The nerve agents soman and sarin were shown to bind covalently to human albumin on tyrosine (Black et al., 1999; Adams et al., 2004) and to be released by treatment with potassium fluoride (Adams et al., 2004).

Albumin has also been demonstrated to be an OP hydrolase, hydrolyzing chlorpyrifos oxon, O-hexyl O-2, 5-dichlorophenylphosphoramidate, and paraoxon at measurable rates (Erdos and Boggs, 1961; Ortigoza-Ferado et al., 1984; Sultatos et al., 1984; Sogorb et al., 1998a). The apparent Km of bovine albumin is 0.41 mM for chlorpyrifos oxon, 1.85 mM for paraoxon (Sultatos et al., 1984) and that of human albumin is 3.6 mM for DFP (Means and Wu, 1979). Despite this seemingly consistent body of results, some issues have been raised regarding the reaction of OP with albumin. It has been questioned whether the observed OP hydrolase activity was associated with the albumin molecule itself, or with minor phosphotriesterase contaminants in the albumin preparation (Erdos and Boggs, 1961). In addition, the possibility has been raised that DFP binds to one site in albumin, but that other OP bind to a different site (Mourik and de Jong, 1978; Sultatos et al., 1984).

We developed a MALDI-TOF assay for identifying DFP-labeled human albumin. In addition we determined the fragmentation pattern for the DFP-labeled human albumin tryptic peptide. Knowledge of the fragmentation pattern will allow development of a highly sensitive selective ion monitoring assay to test for exposure to DFP.

Materials and Methods

Materials. Purified human serum albumin, essentially fatty acid free (Fluka via Sigma, St. Louis, MO; cat no. 05418), pepsin (Sigma, St. Louis, MO; cat no. P6887 from porcine gastric mucosa), modified trypsin, sequencing grade (Promega, Madison, WI; cat no. V5113), diisopropylfluorophosphate (Sigma; cat

no. D0879), acetonitrile (HPLC grade 99% ACROS cat no. 61001-0040 from Fisher Scientific, Pittsburgh, PA), trifluoroacetic acid, sequencing grade (Beckman Instruments, Palo Alto, CA; cat no. 290203), 2,5-dihydroxybenzoic acid (DHBA) matrix (Applied Biosystems Foster City, CA), alpha-cyano 4-hydroxycinnamic acid (CHCA) (Sigma; cat no. 70990) was recrystallized before use. Calibration standards for MALDI-TOF were from New England Biolabs (Beverly, MA; cat no. P7720S). They included Angiotensin 1, 1297.51 amu, ACTH (7-38) 3660.19 amu, and ACTH (18-39) 2466.73 amu. Double distilled water was prepared in-house and autoclaved.

Preparation of DFP-albumin peptic peptides. Fatty acid-free human albumin at a concentration of 10 mg/ml, which is 150 µM, was dissolved in 25 mM ammonium bicarbonate pH 8.6 and treated with an equimolar concentration of DFP for 24 h at 37˚C. The pH of 1000 µl reaction mixture was reduced to pH 2.3 by the addition of 500 µl of 1% trifluoroacetic acid. Pepsin was dissolved in 10 mM HCl to make 1 mg/ml and stored at -80˚C. The albumin was digested with pepsin (1:250 ratio) for 2 hours at 37˚C and diluted to 1 pmol/µl with 0.1% trifluoroacetic acid.

MALDI-TOF. A 1 µl aliquot of diluted peptic digest was applied to a stainless steel target plate, air dried, and overlaid with 1 µl of 2,5-dihydroxybenzoic acid matrix. The CHCA matrix gave similar results. Mass spectra were acquired with the Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA) in linear positive ion mode, 20,000 volts accelerating voltage, 94% grid voltage, 0.1% guide wire, 350 nsec extraction delay time, automated laser intensity adjustment from 1000 to 1600. The instrument was calibrated with a peptide calibration mixture from New England Biolabs. Mass accuracy for each standard was within 0.05% of the corresponding average molecular weight. Spectra were acquired in automatic mode, by examining signals from random spots on a target. The signals from the first 10 spots that met the acceptance criteria were summed into one final profile mass spectrum The acceptance criteria were signal intensities between 1000 and 55000 counts, with signal to noise ratios of 10 or greater, and minimum resolution of 50. The final spectrum was the average of 1000 shots.

The MS-Digest program from the UCSF Mass Spectrometry Facility was used to calculate the masses of the peptic peptides expected from digests of human serum albumin.

Quadrupole mass spectrometer. MS/MS spectra were acquired on a QTRAP 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA) with a nano electrospray ionization source. DFP-labeled albumin digested with trypsin was infused into the mass spectrometer via a fused silica emitter (360 µm o.d., 20 µm i.d., 10 µm taper from New Objective, Woburn, MA), using a Harvard syringe pump to drive a 100 µl Hamilton syringe equipped with an inline 0.25 micron filter, at a flow rate of 1 µl/min. Samples were prepared in 50% acetonitrile, 0.1% formic acid. Positive ion spectra were

obtained. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with a collision energy of 40±5 volts and a nitrogen gas pressure of 4 x 10^{-5} Torr. The final enhanced product ion scan was the average of 212 scans.

Results

Reaction of pure human albumin with DFP. Fatty acid free-albumin was used because fatty acids and DFP bind to the same albumin domain and therefore fatty acids could block the binding of OP (Means and Wu, 1979; Peters, 1996). The covalent attachment site for human albumin, Tyr 411, is located near the surface of the albumin molecule where it is accessible to proteases. Digestion with trypsin at pH 8.6 or with pepsin at pH 2-2.5 released peptides of the expected masses without the need to denature or to reduce and alkylate the disulfide bonds of albumin. Peptides containing Tyr 411 had the sequence YTK (411 m/z, singly charged mass) when the protease was trypsin, and the sequences VRYTKKVPQVSTPTL (1718 m/z) and LVRYTKKVPQVSTPTL (1831 m/z) when the protease was pepsin. Pepsin routinely missed one cleavage in our experiments.

The tryptic YTK peptide (411 m/z) and the DFP adduct had masses that overlapped with matrix peaks, which made them difficult to detect by MALDI mass spectrometry. Furthermore, the YTK peptide and YTK-DFP adduct did not seem to ionize when irradiated by the nitrogen laser in the Voyager DE-PRO, though they did ionize in the electrospray source of the QTRAP. By contrast, the larger peptides produced by digestion of albumin with pepsin separated well from matrix and ionized to give good signals in the Voyager DE-PRO. Therefore, samples intended for analysis by MALDI-TOF were digested with pepsin rather than with trypsin.

Figure 2.2.1 shows the MALDI-TOF spectra obtained for pepsin-digested human albumin before and after treatment with DFP. The top panel shows masses at 1718 and 1831 m/z, which are consistent with the peptides from unlabeled albumin that contain Tyr 411. Additional albumin peptides are also present but they do not contain Tyr 411 and are therefore not discussed. The DFP panel shows two new peaks at 1882 and 1995 m/z for the diisopropoxyphosphate adducts. About 70% of the albumin is labeled. Since the MALDI conditions disrupt non-covalent interactions, the DFP-peptide adducts must be covalently formed. These results support the conclusion that human albumin is labeled by DFP and are consistent with the site for covalent attachment being Tyr 411. The masses correspond to the dialkoxy adducts rather than the monoalkoxy adducts. Masses for monoalkoxy adducts were not found, supporting the conclusion that the DFP-albumin adduct does not age.

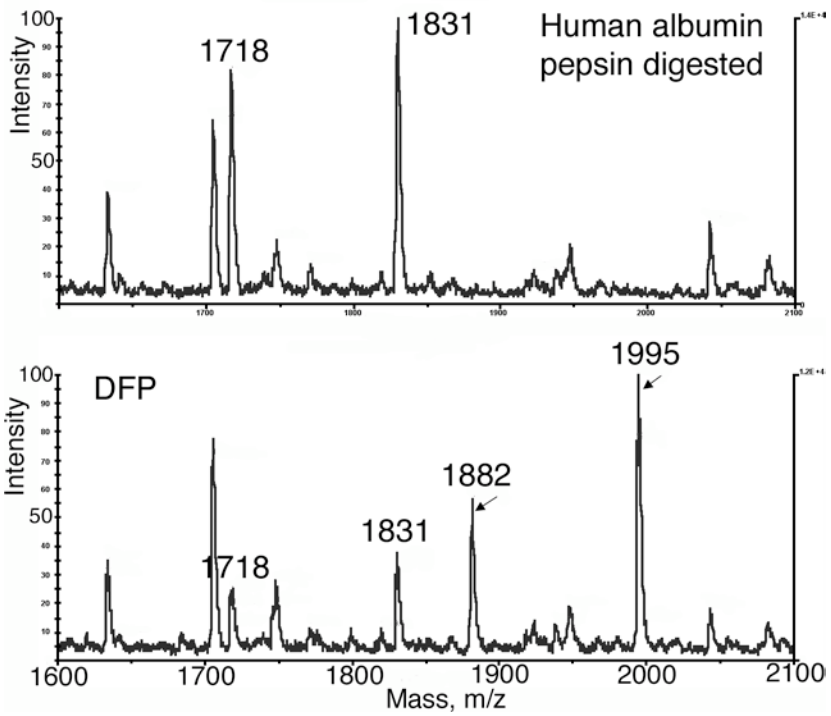

Figure 2.2.1. MALDI-TOF analysis of human albumin-DFP adducts. (Top panel) Digestion of human albumin with pepsin at pH 2.3 yields 2 peptides containing Tyr 411 whose average mass to charge ratios are 1718 and1831 (singly protonated). (DFP panel) Incubation of human albumin with DFP yields diisopropoxyphosphate adducts of 1882 and 1995 m/z. Samples were diluted to 1 pmol/µl before plating 1 µl on the MALDI target with 2,5-dihydroxybenzoic acid matrix.

Saturating the albumin binding sites. Unlabeled 1718 and 1831 m/z peptides were always present when the concentration of DFP was the same as the concentration of albumin. However, when the DFP concentration was 40-fold higher than the albumin concentration, all the Tyr 411 sites were occupied and no unlabeled peptides of 1718 and 1831 m/z were detected. DFP labeling reactions with albumin were performed at pH 8.5 because labeling occurs at high pH but is markedly decreased at neutral pH (Murachi, 1963; Means and Wu, 1979).

Limit of detection. It was essential to dilute the albumin and plasma digests before applying the sample on the target plate. Undiluted samples did not show the desired peptides due to ion suppression and charge competition effects. The limit of detection of DFP-labeled peptide was determined from dilutions of peptides in which all of the Tyr 411 sites were occupied. The diluent was 0.1%

trifluoroacetic acid in water. Peaks of interest were detected after 100, 1000, 3000, and 9,000 fold dilution of a sample whose starting concentration was 600 µM (40 mg/ml) albumin. The signal to noise ratio for the 1:9000 diluted sample was 3:1. At 18,000 fold dilution the peak height was only two fold above the noise. Thus, the minimum detectable signal from a DFP-labeled peptide occurred at 0.07 pmol/µl.

MS/MS analysis confirms DFP binding to Tyr 411 of the YTK peptide. DFP-labeled human albumin was digested with trypsin and the digest was infused into the QTRAP mass spectrometer. The enhanced mass spectrum showed a peak at 575.4 m/z, which is consistent with the singly charged YTK peptide covalently bound to DFP ($[M+H]^{+1}$ = 411 amu for the YTK peptide plus 164 amu added mass from DFP). This peptide was subjected to collision-induced dissociation with nitrogen as the collision gas. The resulting enhanced product ion spectrum yielded amino acid sequence information consistent with the sequence YTK where DFP is covalently bound to tyrosine. See Figure 2.2.2. A mass was found at 147.2 amu, indicative of the C-terminal lysine from a y-series. This was followed by masses at 248.3, for the ThrLys dipeptide, and at 575.4, for the Tyr*ThrLys tripeptide, including the N-terminus, from a y-series (where Tyr* represents the diisopropylphospho-adduct of tyrosine). No relevant signals were found at higher mass. No evidence for the diisopropylphospho-adduct of threonine was found.

Further, convincing evidence for covalent binding of DFP to Tyr 411 comes from the presence of 6 masses that are all consistent with various fragments of DFP attached to tyrosine, alone or in conjunction with the YTK peptide. The structures of these 6 ions are shown in Figure 2.2. As mentioned before, the ion at 575.4 amu is consistent with the singly protonated YTK peptide plus the added mass from covalent attachment of DFP. Neutral loss of 42 amu yields the 533.4 amu ion. Loss of 42 amu is predicted for b-elimination of propylene from the diisopropylphosphate adduct. This b-elimination type reaction, also referred to as a McLafferty rearrangement (McLafferty, 1959; Fredriksson et al., 1995) is a facile reaction commonly seen during collision induced dissociation of phosphopeptides (McLachlin and Chait, 2001). A second neutral loss of 42 amu yields the 491.3 amu ion; this mass is consistent with a phosphorylated YTK peptide. In theory, all three of these masses are consistent with DFP adducts of either tyrosine or threonine. However, the mass at 244.2 amu is characteristic of an N-terminal phosphotyrosine, b-series aziridone ion, and the 226.2 amu ion is consistent with its dehydration product (Mann et al., 2001). In addition, the mass at 216.2 amu is characteristic of a phosphotyrosine immonium ion (Steen et al., 2001). Furthermore, no indication of phosphorylated or organophosphorylated threonine was found. These results prove that DFP covalently binds to Tyr 411 of human albumin.

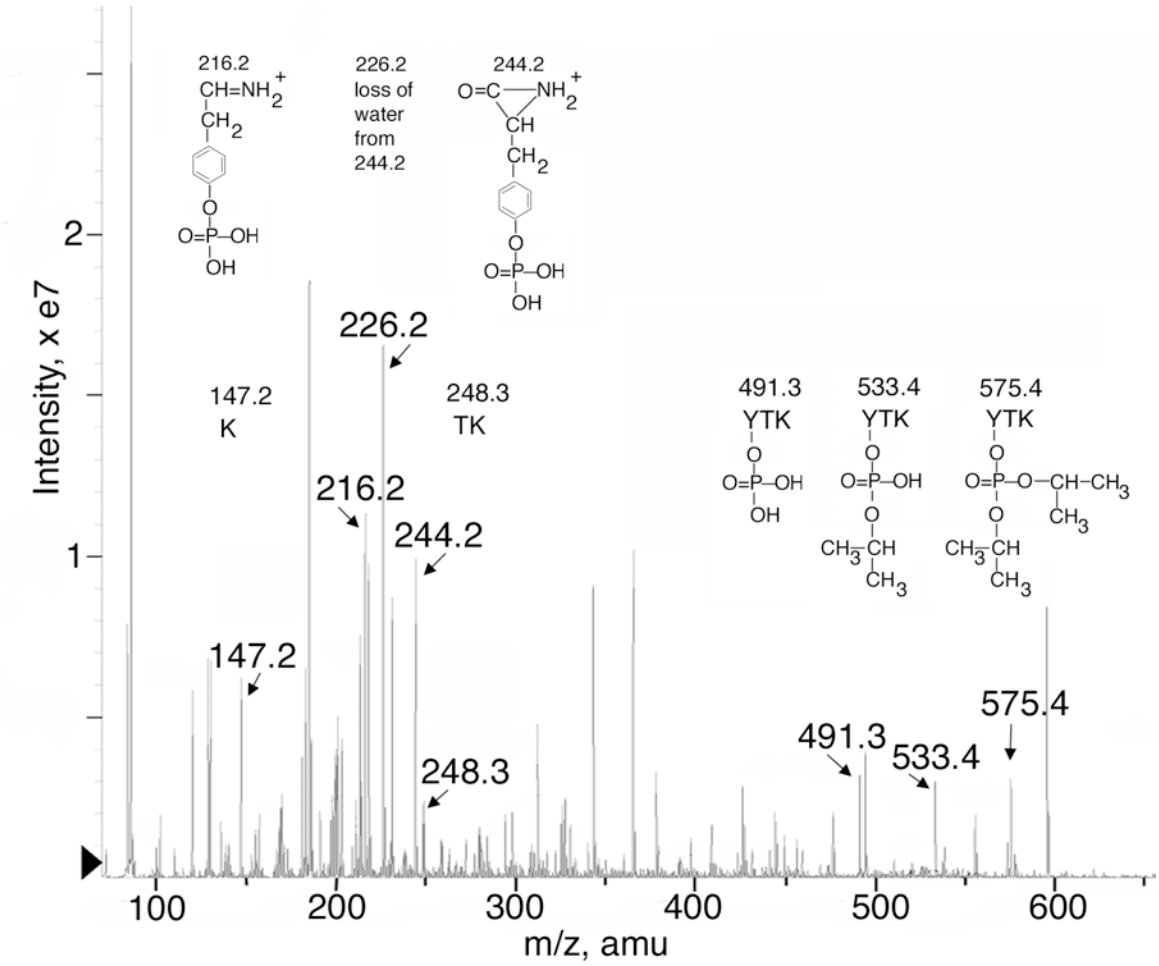

Figure 2.2.2. Product ion spectrum of DFP-labeled human albumin peptide of mass 575.4. DFP-labeled albumin was digested with trypsin and infused into the QTRAP mass spectrometer. The singly charged parent ion of 575.4 m/z has the sequence YTK and has the diisopropylphosphate group covalently bound to tyrosine. Six fragment masses and their corresponding structures are shown.

No evidence for any form of the dephosphorylated YTK peptide was found. This probably reflects the relative difficulty of releasing OP from tyrosine, compared to the other fragmentation pathways available to the diisopropylphospho-YTK peptide. Facile dephosphorylation of phospho-tyrosine via a beta-elimination type mechanism is not available because tyrosine does not afford a suitable environment. Beta-elimination would require the shift of a proton from the beta carbon of the leaving group to the phosphate oxygen, with concomitant formation of a double bond between the alpha and beta carbons of the leaving group. For phospho-tyrosine, the beta-proton of the leaving tyrosine is aromatic and therefore not readily released; furthermore, formation of the

double bond requires introduction of a triple bond into the aromatic ring of tyrosine, another difficult operation. Though phosphate can be released from tyrosine during collision induced dissociation, it seems to require the presence of hydroxyl moieties on the phosphate. Even then, the yield of dephosphorylation is poor (Fredriksson et al., 1995; Tholey et al., 1999). Thus, it would appear that elimination of propylene from the diisopropoxy-moieties and fragmentation of the peptide backbone provide energetically more acceptable routes for utilization of the collision energy than dephosphorylation of the tyrosine.

The presence of two isopropyl groups in parent ion 575.4 supports the conclusion that the DFP-albumin adduct does not age.

The unique set of 6 phosphorylated fragment ions in Figure 2.2.2 for DFP-albumin could be useful for identifying exposure to DFP in a mass spectrometry method that selectively searches for characteristic fragment ions.

Discussion

Mechanism of OP labeling of albumin. DFP was found to label Tyr 411 of human albumin. The stoichiometry of labeling has been shown to be one mole ^{3}H-DFP or ^{14}C-DFP incorporated per mole of albumin (Murachi, 1963; Means and Wu, 1979; Hagag et al., 1983; Sultatos et al., 1984). The specific labeling of one tyrosine in a molecule that contains 18 tyrosines suggests that Tyr 411 is in a special environment. This tyrosine has an unusually low pKa of 7.9 - 8.3 (Means and Wu, 1979; Ahmed et al., 2005), in contrast to the pKa of 10 for the average tyrosine.

Tyr 411 is the active site residue not only for reaction with OP, but also for reaction with esters such as p-nitrophenyl acetate, carbamates such as carbaryl, and amides such as o-nitroacetanilide (Means and Wu, 1979; Sogorb et al., 1998b; Sogorb et al., 2004; Manoharan and Boopathy, 2006). The esterase and amidase activity of albumin can be inhibited by pretreatment with DFP. Conversely, labeling with DFP can be prevented by pretreatment with p-nitrophenyl acetate, which forms a stable acylated albumin adduct (Means and Wu, 1979). The sensitivity of albumin esterase activity to ionic strength led Means and Wu to conclude that the reactive tyrosine residue is located on the surface of albumin in an apolar environment adjacent to several positively charged groups. This description of the OP binding site of albumin was proven to be correct when the crystal structure was solved (He and Carter, 1992; Sugio et al., 1999). Subdomain IIIa of albumin contains a pocket lined by hydrophobic side chains. The hydroxyl of Tyr 411 is close to the side chains of Arg 410 and Lys 414.

Site-directed mutagenesis experiments have shown that albumin esterase activity is abolished when Tyr 411 is mutated to Ala, and severely diminished when Arg 410 is mutated to Ala (Watanabe et al., 2000). These results support Tyr 411 as the active site for albumin esterase activity and support a role for Arg 410 in stabilizing the reactive anionic form of Tyr 411. The negatively charged Tyr 411 is available for nucleophilic attack on ester and amide substrates.

Though crystal structures of several ligand albumin complexes have been solved (Ghuman et al., 2005), the crystal structure of an OP-albumin adduct is not yet available.

No aging of OP-albumin adducts. Aging of OP-labeled acetylcholinesterase and butyrylcholinesterase is defined as the loss of an alkoxy group from the OP-labeled active site serine (Benschop and Keijer, 1966; Michel et al., 1967). The nerve agents sarin, soman, and VX yield the same aged OP derivative, so that these agents may be difficult to distinguish when bound to acetylcholinesterase or butyrylcholinesterase (Millard et al., 1999a; Millard et al., 1999b).

The covalent bond between DFP and the active site serine of acetylcholinesterase and butyrylcholinesterase undergoes aging. However DFP bound to albumin does not age. Aging is a catalytic process that requires the participation of nearby histidine and glutamic acid residues (Kovach et al., 1997). Residues that promote aging are not present in the active site pocket of albumin. The absence of aging allows the bound OP to be spontaneously released from Tyr 411. This makes albumin an OP hydrolase, though a very slow one. Albumin hydrolyzes O-hexyl O-2,5-dichlorophenylphosphoramidate, chlorpyrifos oxon, and paraoxon (Erdos and Boggs, 1961; Ortigoza-Ferado et al., 1984; Sultatos et al., 1984; Sogorb et al., 1998a).

Antibody to OP-albumin. Our results suggest the possibility of detecting OP exposure through use of an antibody detection assay directed toward the OP-albumin adduct at Tyr 411. There is precedent for the generation of antibodies to very small haptens bound to protein. For example, antibodies that distinguish between phosphotyrosine, phosphoserine, and phosphothreonine have been successfully produced (Glenney et al., 1988; Levine et al., 1989). Antibodies to soman, sarin, and VX bound to carrier proteins through a chemical linker have been produced (Grognet et al., 1993; Zhou et al., 1995; Johnson et al., 2005) . The proposed OP-albumin epitope could be more useful for detection of OP exposure than existing antibodies because the OP-albumin adduct has no chemical linker and no foreign protein environment.

An OP-albumin adduct at Tyr 411 may generate an antibody response in exposed individuals and the antibody could be detected to determine a history of exposure to OP. This would facilitate monitoring exposure to OP long after the exposure incident and long after the antigen has disappeared.

A copy of a publication that reports these results is attached.

Bin Li, Lawrence M. Schopfer, Steven H. Hinrichs, Patrick Masson, Oksana Lockridge (2007) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr 411. Analytical Biochemistry. *Anal Biochem* **361**:263-272.

TASK 2

2.3. Label pure bovine trypsin with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation.
reported July 20, 2007

Relation to statement of work. Results for task 2.3 are reported.

The active site serine of DFP-labeled bovine trypsin loses DFP and water in the collision chamber of the mass spectrometer to yield dehydroalanine

Summary

The purpose of this work was to determine whether the DFP-labeled active site peptide of trypsin behaves similarly to OP-labeled BChE peptides in the mass spectrometer. Both trypsin and BChE have an active site serine that is covalently modified by OP. No other residue in trypsin and BChE is modified by OP. Bovine trypsin was reacted with DFP, denatured in 8 M urea, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with porcine trypsin. The active site peptide was isolated by HPLC and infused into the QTRAP 4000 mass spectrometer where MS/MS spectra showed dehydroalanine ions. The most intense ions lost the entire OP and in addition lost a water molecule to yield dehydroalanine in place of the active site serine. It was concluded that there is a consistent pattern of fragmentation independent of the identity of the protein, of the OP, and of the protease used for digestion, but dependent on the presence of an active site serine.

Introduction

Our goal is to find a pattern in the fragmentation mass spectrum of OP-labeled peptides, for the purpose of aiding in the identification of OP exposure. From our work and that of others (Fidder et al., 2002; Schopfer et al., 2005; Tsuge and Seto, 2006; Sun and Lynn, 2007) we expect the OP-labeled active site serine to be degraded to dehydroalanine in the collision chamber of the mass spectrometer during elimination of the OP. This mechanism is analogous to phosphate elimination from phosphorylated serine and threonine

phosphoproteins (Neubauer and Mann, 1999). In contrast, phosphotyrosine does not undergo this beta-elimination mechanism (Schopfer et al., 2005).

Materials and Methods

Bovine trypsin TPCK treated, type XIII from bovine pancreas, essentially salt-free, catalog number T-8642 from Sigma. Accession # gi:559311, MW 24,800. Diisopropyl fluorophosphate (DFP) is a liquid with a concentration of 5.73 M (D-0879, Sigma, St. Louis, MO). A 0.3 M stock solution of DFP was prepared in ethanol and stored at -80˚C. Porcine trypsin sequencing grade was from Promega.

Reaction of trypsin with DFP. 10 mg of lyophilized trypsin dissolved in 1.5 ml of 50 mM ammonium bicarbonate was mixed with 1 µl of 5.73 M DFP. The final concentrations were 268 µM trypsin and 3280 µM DFP. After 1.5 hours at 37˚C the solution became turbid. The solution was clarified by denaturing in 8 M urea, adding dithiothreitol to 10 mM and heating in a boiling water bath for 10 min. Free sulfhydryls were carbamidomethylated with 100 mM iodoacetamide.

Tryptic peptides. The DFP-labeled, carbamidomethylated bovine trypsin was dialyzed against 3 x 4 Liters of 10 mM ammonium bicarbonate to remove excess DFP and salts. A 1 mg aliquot of the DFP-trypsin was digested with 0.02 mg of porcine trypsin for 24 h at 37˚C. Peptides were purified on a Phenomenex Prodigy 5 micron ODS(2), 100 x 4.60 mm column on a Waters HPLC system. The column was equilibrated with 0.1% TFA and eluted with a gradient increasing in acetonitrile to 60% in 60 min, at a flow rate of 1ml/min. One ml fractions were collected. A 0.5 µl aliquot from each fraction was analyzed on the MALDI-TOF to identify fractions containing the labeled peptide. The DFP-labeled peptide of mass 1773.7 was in fraction 19. The sample was dried under vacuum and dissolved in 100 µl of 50% acetonitrile, 0.1% formic acid in preparation for infusion into the mass spectrometer.

QTRAP 4000 mass spectrometer. MS/MS spectra were acquired on a QTrap 4000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA) with a nano electrospray ionization source. DFP-labeled BChE digested with trypsin was infused into the mass spectrometer via a fused silica emitter (360 µm o.d., 20 µm i.d., 10 µm taper from New Objective, Woburn, MA), using a Harvard syringe pump to drive a 100 µl Hamilton syringe equipped with an inline 0.25 micron filter, at a flow rate of 0.35 µl/min. Positive ion spectra were obtained. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with a collision energy of 40-50 volts and a nitrogen gas pressure of 4×10^{-5} Torr. The final enhanced product ion spectra were the average of 200-250 scans.

Results

The active site tryptic peptide of bovine trypsin contains 16 amino acids. Residue 7 is the active site serine in the consensus sequence GXSXG. The peptide mass of 1773.7 includes 164 amu from DFP and 2 x 57 amu from the 2 carboxymethyl groups on the two cysteines. Table 2.3.1. The peptide mass is 1731.7 after aging, that is after loss of one isopropyl group from DFP.

Table 2.3.1. Expected masses of the bovine trypsin active site peptide labeled with DFP and carbamidomethylated. Accession gi:559311.

sequence	no label	+164 DFP	+ 122 DFP aged
DSCQGDSGGPVVCSGK	1609.7	1773.7	1731.7

The partially purified DFP-labeled active site peptide was infused into the QTRAP 4000 mass spectrometer. Figure 2.3.1 shows the MS/MS spectrum for the doubly charged parent ion 887.5.

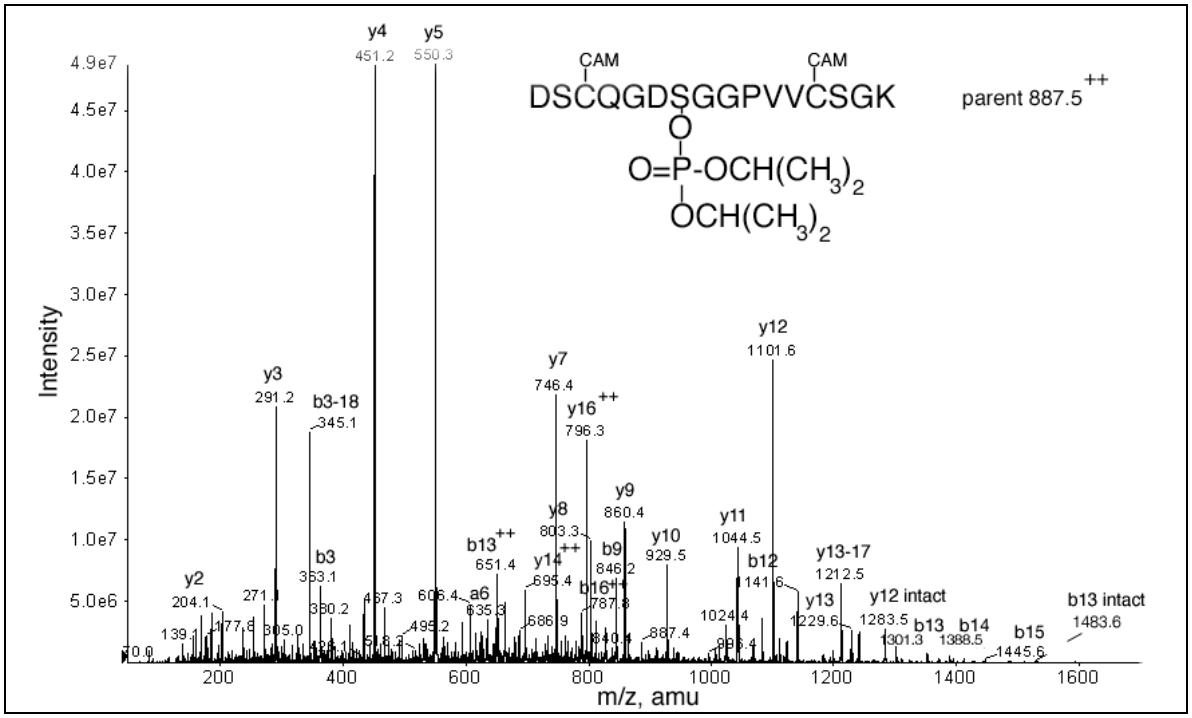

Figure 2.3.1. MS/MS spectrum of DFP-labeled bovine trypsin carbamidomethylated on cysteine. The doubly charged parent ion has a mass to charge ratio of 887.5.

The MS/MS spectrum in Figure 2.3.1 supports the amino acid sequence DSCQGDSGGPVVCSGK and confirms that the DFP labeled residue is serine 7. The diisopropylphosphate structure was found on two ions, singly charged y12

and b13. These ions had weak but detectable signals. All other ions that included residue 7 had a mass that was smaller by 18 amu than expected for serine. The active site serine had lost water and become dehydroalanine. Table 2.3.2 lists the 18 dehydroalanine ions present in Figure 2.3.1. Dehydroalanine in place of serine is a signature for OP-labeled serine.

Table 2.3.2. Dehydroalanine fragment ions in Figure 2.3.1.

+1	mass	+1	mass	+2	mass	+2	mass
b9	846.2	y10	929.5	b13	651.4	y14	695.4
b12	1141.6	y11	1044.5	b16	787.8	y16	796.3
b13	1301.3	y12	1101.6				
b14	1388.5	y13	1229.6				
b15	1445.6	y13 -17	1212.5				

Discussion

Pattern of fragmentation of OP-labeled serine peptides. Comparison of MS/MS spectra of OP labeled BChE, trypsin, chymotrypsin, and albumin shows a common pattern (Table 2.3.3). Collision induced dissociation always releases the OP and a water molecule from the active site serine. The daughter ions contain dehydroalanine in place of the active site serine. This pattern is independent of the identity of the OP-labeled protein, though the protein must contain a serine capable of covalent binding to OP. The pattern is also independent of the OP and of the protease used for digestion.

Table 2.3.3. MS/MS fragmentation pattern of OP-labeled serine.

OP-labeled protein	peptide sequence	OP	protease	reference	pattern
human BChE	svtlfgeSagaasvslhllspgshslftr	DFP	trypsin	Lockridge, this report	dehydroAla
human BChE	svtlfgeSagaasvslhllspgshslftr	FP-biotin	trypsin	Lockridge, unpublished	dehydroAla
human BChE	svtlfgeSagaasvslhllspgshslftr	soman	trypsin	Lockridge, unpublished; (Fidder et al., 2002)	dehydroAla; +78
human BChE	svtlfgeSagaasvslhllspgshslftr	sarin	trypsin	Lockridge, unpublished	dehydroAla
human BChE	fgeSagaas	sarin	pepsin	(Fidder et al., 2002)	dehydroAla
human BChE	fgeSagaas	dimethyl paraoxon	pepsin	(Fidder et al., 2002)	dehydroAla
human BChE	fgeSagaas	diethyl paraoxon	pepsin	(Fidder et al., 2002)	dehydroAla
human BChE	geSagaasvsl	VX	chymotrypsin	(Tsuge and Seto, 2006)	dehydroAla

human BChE	geSagaasvsl	soman	chymo trypsin	(Tsuge and Seto, 2006)	dehydroAla
human BChE	geSagaasvsl	sarin	chymo trypsin	(Tsuge and Seto, 2006)	dehydroAla
bovine chymo trypsin	damicagasgvsscmg dSggplvck	DFP	trypsin	(Tsuge and Seto, 2002)	dehydroAla; +164 peaks
bovine trypsin	dscqgdSggpvvcsgk	DFP	trypsin	Lockridge, this report	dehydroAla
bovine trypsin	dscqgdSggpvvcsgk	FP-biotin	trypsin	(Schopfer et al., 2005)	dehydroAla
equine BChE	svtlfgeSagaasvslhllspr	dimethyl paraoxon	trypsin	(Sun and Lynn, 2007)	dehydroAla
equine BChE	svtlfgeSagaasvslhllspr	diethyl paraoxon	trypsin	(Sun and Lynn, 2007)	dehydroAla
equine BChE	svtlfgeSagaasvslhllspr	EPN oxon	trypsin	(Sun and Lynn, 2007)	dehydroAla
human albumin	aefaevSk	FP-biotin	trypsin	Lockridge, this report	dehydroAla; +164 peaks
human BChE	geSagaasvsl	no label	chymo trypsin	(Tsuge and Seto, 2006)	dehydroAla

The MS/MS spectra of DFP-labeled chymotrypsin and of FP-biotin labeled albumin included y-ions that carried the +164 mass of DFP as well as y-ions that had lost the OP and become dehydroalanine. Fidder's soman-labeled BChE fragmented to a mixture of y-ions, some of which still carried +78 from aged soman, and others that had converted to dehydroalanine. Under our conditions, soman-labeled BChE fragmented almost exclusively to dehydroalanine. We think a high collision energy eliminates the OP to yield dehyroalanine, whereas a low collision energy does not break the OP-serine bond.

Loss of water from serine can also occur when the serine carries no label. The unlabeled active site chymotryptic peptide of human BChE fragmented to a mixture of dehydroalanine b-ions and standard b-ions (Tsuge and Seto, 2006). Therefore dehydroalanine ions by themselves are not evidence of exposure to OP. A second requirement is a parent ion mass that includes the mass of the OP.

It is concluded that an OP-labeled serine peptide has the following signature: 1) a parent ion mass that includes the mass of the peptide and the OP, and, 2) MS/MS fragment ions that have dehydroalanine in place of serine. This knowledge is useful for identification of OP exposure by mass spectrometry.

TASK 2

2.4. Identify the proteins in human blood that bind DFP.

final report 20 Nov 2007

Relation to statement of work. Results for task 2.4 are reported.

DFP-labeled proteins in human plasma

Summary

The goal was to identify proteins in human plasma modified by DFP. Proteins were separated into high and low abundance fractions with an antibody column. Both fractions were treated with 20-40 mM DFP, dialyzed to remove excess DFP, reduced, alkylated, and digested with trypsin. Peptides were separated on a strong cation exchange HPLC column, followed by reverse phase HPLC and analysis on a QTRAP 2000 mass spectrometer. MS/MS spectra were analyzed by Mascot and OMMSA software to identify DFP-labeled proteins. The top hits identified by the search engines were evaluated. The proteins in which we have the most confidence as having been labeled with DFP are albumin and apolipoprotein. We had expected to find DFP-labeled butyrylcholinesterase, but the labeled active site peptide was not found. A strategy that enriches for butyrylcholinesterase is more likely to succeed.

Introduction

Our work in Tasks 2.1 and 2.2 had shown that DFP labels human butyrylcholinesterase and human albumin and that the DFP-labeled peptides can be identified by mass spectrometry. It was anticipated that additional plasma proteins would also be labeled with DFP. Our goal was to identify these additional proteins.

The strategy we adopted was one successfully used by the laboratory of Richard Smith at Pacific Northwest Laboratories to identify proteins labeled by nitrous oxide (Sacksteder et al., 2006). Tryptic peptides from the entire plasma proteome were prepared and separated by strong anion exchange into 23 fractions. Each fraction was analyzed by LC/MS/MS. The 25,000 MS/MS spectra were analyzed by Mascot and OMMSA software.

Materials and Methods

Reagents. Beckman Coulter ProteomeLab IgY-12 HC spin column depletion kit depletes human plasma of the 12 most abundant proteins: albumin, IgG, alpha1-

antitrypsin, IgA, IgM, transferrin, haptoglobin, alpha1-acid glycoprotein (orosomucoid), alpha2-macroglobulin, apolipoproteins AI and AII of HDL, and fibrinogen. Product #A24618.

Depletion of 12 most abundant proteins. The spin column for depletion of 12 most abundant proteins is designed to process 20 µl of plasma. We reused the spin column 10 times to process 200 µl of human plasma. The low abundance proteins had a protein concentration of 4.8 µg/µl in 500 µl. The high abundance proteins had a protein concentration of 12 µg/µl in 400 µl. As expected, BChE activity was found in the low abundance proteins but not in the high abundance proteins.

DFP labeling. 1200 µg of low abundance proteins in 250 µl and 1600 µg of the high abundance proteins in 133 µl were treated with 1 µl of 5.7 M DFP to give final concentrations of 23 and 43 mM DFP. Samples were incubated at 37˚ for 16 h.

Controls. The control samples were treated identically to the DFP labeled samples with the difference that they were not treated with DFP.

Reduction, alkylation, and trypsin digestion. Samples were denatured in 8 M urea, reduced with 5 mM dithiothreitol, alkylated with 40 mM iodoacetamide, and dialyzed against 4 x 4 liters of 10 mM ammonium bicarbonate. Proteins were digested with sequencing grade trypsin (Promega) at a ratio of 100:1 (wt:wt) at 37˚C for 48 h.

Off-line strong cation exchange HPLC. Tryptic peptides were fractionated by strong cation exchange chromatography on a Polysulfoethyl A 200 mm x 2.1 mm column (PolyLC, Columbia, MD) protected by a guard column. The separations were performed at a flow rate of 0.25 ml/min on a Waters HPLC system. The mobile phases consisted of 10 mM ammonium formate pH 3.0/25% acetonitrile (A), and 500 mM ammonium formate pH 6.8/25% acetonitrile (B). After the sample was loaded, the column was washed for 30 min with A, followed by a gradient from 0-50% B in 40 min, and 50 to 100% B in 10 min. The protein concentration in each fraction was measured in a microtiter plate assay with Coomassie blue, and fractions were pooled so that the sample for mass spectrometry contained approximately 5 µg. Fractions were dried under vacuum and dissolved in 100 µl of 5% acetonitrile, 0.1% formic acid for analysis on the mass spectrometer. A total of 23 fractions were analyzed on the QTRAP 2000 mass spectrometer: 8 from the high abundance proteins, and 15 from the low abundance proteins.

LC/MS/MS analysis. A Dionex Ultimate 1000 HPLC system (Chicago, IL), equipped with two pumps, a vacuum degasser, auto-sampler, and controller module, was used in this study. Chromatographic separation was performed on a Vydac C18 polymeric nanocolumn (#218MS3.07515, P.J. Cobert Associates, St.

Louis, MO)) at room temperature. The mobile phases were water and acetonitrile, both containing 0.05% formic acid. The gradient program, with a total run time of 120 min, started with a 30 min wash of the 5 µg sample to remove salt. Peptides were eluted with a gradient increasing from 5 to 50% acetonitrile in 60 min, followed by re-equilibration for 30 min. The flow rate remained at 0.3 mL/min throughout the run.

A triple quadrupole tandem mass spectrometer QTRAP 2000 instrument (ABI-SCIEX, Foster City, CA) equipped with nanospray was operated in positive ionization mode. The Analyst 1.4 software package was used for instrument control and data acquisition. Briefly, the mass spectrometer was operated in the data-dependent mode to automatically switch between MS, Enhanced resolution scan and MS/MS. MS spectra from m/z 150-1575 were acquired and the top 3 most intense ions were sequentially isolated for an enhanced resolution scan and then simultaneously fragmented in the ion trap using collision-induced dissociation at a target value of 10, using nitrogen as the collision gas.

Data Analysis

All MS/MS spectra were searched against the human International Protein Index data base (consisting of 49,161 protein entries, Version 3.05, April, 2005; available online at www.ebi.ac.uk/IPI) using Mascot (Matrix Science) and OMSSA (NCBI) search algorithms. Carbamidomethylation of cysteine was selected as the fixed modification. Oxidation of methionine and organophosphorylation of serine, threonine, or tyrosine by DFP were selected as variable modifications. The added mass value for diisopropylphosphate is 164.0602 (monoisotopic mass) or 164.134 (average mass), while the added mass for monoisopropylphosphate, the aged form of DFP, is 124.0289 (monoisotopic mass) or 124.072 (average mass).

The following steps were used for the search. The MS/MS data were extracted from the raw data using PERL script and merged into one file. The file was converted into an MGF file readable by OMMSA. The MGF file was analyzed by OMMSA against the reference IPI human protein database. About 17,000 peptides were exported in CSV format to the ACCESS program in Microsoft Office. A small subset of about 120 peptides, those containing a modification, was placed into a new file. The MS/MS spectrum of each DFP-modified peptide was manually inspected to determine the quality of the data. Only those DFP-peptides that had either a continuous y-ion or b-ion series were accepted. Each of the accepted DFP-peptides was checked against the control set of peptides to make certain it was absent in the control set.

Results

Peptides from the high abundance fraction of human plasma were separated by strong cation exchange HPLC and reverse phase HPLC before they were fragmented in the QTRAP 2000 mass spectrometer. The MS/MS data were

analyzed by Mascot and OMMSA software to identify DFP-labeled peptides. The DFP-labeled peptides in the high abundance fraction of human plasma are listed in Table 2.4.1.

Table 2.4.1. DFP-labeled tryptic peptides from high abundance human plasma proteins identified by mass spectrometry. The software suggests that one or more Tyrosine, Serine, or Threonine residues are labeled, though we disagree with the software analysis.

sequence	protein name	accession #	Mascot score	labeled residue	DFP
KYLYEIAR	albumin	CAA1491	16	Tyr 2	+164
DYVSQFEGSALGK	apolipoprotein	gi: 178775		Tyr 2	+164
QERSVPTFK	DNA replication factor CDT1	AAG45181.1	19	Ser 4	+122
LAKTYKTTLEK	albumin	AAN17825	20	Thr 4	+164
STQEKLSSR	tousled-like kinase 1	AAF03094.1	20	Ser 1 Ser 8	+122 +122
HPPVSPGRTEK	ankyrin-2	Q01484	9	Thr 9	+122
RPRSAELSEDDLLSQYSLSFTK	exonuclease-I	Q9UNW0	18	Ser 14 Tyr 16 Ser 17 Thr 21	+122 +122 +122 +164
VTVSSASTKGPSVFPLAPSSK	immunoglobulin heavy chain	AAS86000	29	Thr 2 Ser 4	+164 +164
GLQWVSSLFLVSGRTQVAGFAAGRFSFTR	immunoglobulin heavy chain variable region	CAB87536	21	Ser7 Thr 15 Ser 26	+164 +164 +122
TIVSTAQISESR	titin protein	CAA62188	18	Thr 1 Ser 4 Ser 9	+164 +122 +164
TESVTSGPMSPEGSPSK	adducin	AAH65525	12	Ser 3 Ser 6 Ser 16	+122 +122 +122
TEKHSPVSPSAKTER	isoform 1 of ankyrin-2	EAX06290	13	Ser 10 Thr 13	+164 +164

A residue labeled with diisopropylphosphate has an added mass of +164, while a residue labeled with monoisopropylphosphate (the aged form of DFP) has an added mass of +122. We have 100% confidence that the KYLYEIAR peptide of albumin and the DYVSQFEGSALGK peptide of apolipoprotein are labeled with DFP, but have no confirmation that the other assignments are correct.

The low abundance fraction of human plasma proteins was analyzed in separate LC/MS/MS runs. The DFP-labeled peptides are in Table 2.4.2.

Table 2.4.2. DFP-labeled tryptic peptides from the low abundance fraction of human plasma proteins, identified by mass spectrometry.

sequence	protein name	accession #	OMMSA score	labeled residue	DFP
NGNVRNTLLQSK	testis-specific basic protein Y2	NP_004669	5.560e-001	Ser 11	+164
LYIHRVTLR	FLJ16165 phosphatase	NP_001004318	4.063e-001	Tyr 2	+164
MSIDVVEKTNKIK	ATP-binding cassette	NP_000341	3.022e-001	Thr 9	+164
EEDKVGGTPSR	synaptopodin 2	NP_597734	5.257e-001	Thr 8 Ser 10	+164 +164
TSNVLTLSLKR	ubiquitin specific protease 36	NP_079366	8.471e-001	Thr 1 Ser 2	+164 +164
DLGHNDKSSTPGLK SNTPTPR	transducin-like enhancer protein 3	NP_065959.1	8.853e-001	Ser 15 Thr 17	+164 +164
EYTSAVTELVK	cancer susceptibility candidate 1	NP_060742.3	4.429e-001	Tyr 2 Thr 3 Ser 4	+122 +164 +164
LPEGYLEKLTLNSPIF DKPLSR	PCTAIRE protein kinase 1	NP_006192	3.306 E-001	Tyr 5 Thr 10	+164 +122
TAQSPAMVGSPIRSP K	RNApolymerase II transcription factor TAFII 140	NP_114129	3.054e-001	Thr 1 Ser 14	+164 +122
TINSRIGNTVYLTK	ADAMTS-like 3 metalloprotease	NP_997400	2.866e-001	Ser 4 Tyr 11 Thr 13	+122 +164 +122
ESEALGGPGQVLICA SDDR	DNA repair protein	NP_005227	6.085e-001	Ser 2 Ser 16	+164 +164
LSGSSPFSSAFNSLS LDK	hypothetical protein LOC57523	NP_079357.2	8.949e-001	Ser 2 Ser 5 Ser 8 Ser 9	+164 +164 +164 +164
SQPLSSSFHDILSPC K	hypothetical proteinFLJ46819	Q6PFW9	5.536e-001	Ser 1 Ser 13	+164 +164
LETSMLHWTRQIK	dynein heavy chain domain 3	NP-065928	5.264e-001	Thr 3 Thr 9	+164 +164
MSSSSPTGQIASAAD IK	sine oculis homeobox homolog 4	NP_059116	6.347e-001	Ser 3 Ser 5 Thr 7 Ser 12	+164 +164 +164 +164
NNLTILQRYMSSK	RNA methyl transferase domain	NP_060289	8.084e-001	Thr 4 Ser 12	+164 +164

In the Discussion section we explain why we have no confidence in most of the proteins identified by the software as DFP-labeled. However, we do have complete confidence that human apolipoprotein is labeled on Tyr 172 and Tyr 35 in the peptide sequences shown in Table 2.4.3.

Table 2.4.3. Human apolipoprotein peptides labeled with FP-biotin on Y172 or with DFP on Y35. From human plasma.

sequence	gi #	OP binding site
THLAPY*SDELR	178775	Tyr 172
DY*VSQFEGSALGK	178775	Tyr 35

Discussion

Evaluation of high abundance DFP-labeled peptides in Table 2.4.1. The human plasma proteome database is a compendium of 3778 (http://www.plasmaproteomedatabase.org) nonredundant proteins found in human plasma. The database was searched for the proteins in Table 2.4.1. All except DNA replication factor CDT1, tousled-like kinase, and adducin were in the plasma database. Their absence from the database makes it doubtful that DNA replication factor CDT1, tousled-like kinase, and adducin have been correctly identified as DFP-labeled proteins in plasma.

We are certain that albumin is labeled by DFP (see Task 2.2). Labeling of Tyr 138 in peptide KYLYEIAR has been confirmed by us with FP-biotin and chlorpyrifos oxon in Task 1.2.

However, albumin peptide LAKTYKTTLEK with an added mass of +164 on threonine has not previously been identified as OP labeled. An OP-labeled threonine would be expected to fragment to dehydroalanine, but no dehydroalanine peaks are present in the MS/MS spectrum. Therefore, we conclude that albumin peptide LAKTYKTTLEK has been incorrectly identified by the search engine as labeled by DFP.

The MS/MS spectrum for apolipoprotein peptide DYVSQFEGSALGK had a complete series of y6-y11 ions, as well as a-ions and b-ions whose masses exactly matched those expected for the DFP-labeled peptide. All major peaks in the spectrum were assigned. We have complete confidence that this apolipoprotein peptide is labeled on Tyrosine 35.

We doubt that other proteins in Table 2.4.1 are labeled by OP. We are skeptical that a peptide of 29 residues or less can be labeled on 2 or 3 threonines and serines by DFP. There is no precedent in the literature for OP labeling on nearby threonines and serines. None of the DFP-labeled serines in Table 2.4.1 lies within the consensus sequence GXSXG for a serine hydrolase. However, the phosphorylation literature does show that multiple sites within a short distance of each other can become phosphorylated on Serine, Threonine, or Tyrosine by high energy nucleotides ATP or GTP, catalyzed by specific kinases.

Another reason for skepticism is that many of the residues in Table 2.4.1 are labeled with the aged form of DFP, that is with monoisopropylphosphate (+122) rather than with diisopropylphosphate (+164). Aging is a catalytic process. It seems unlikely that the proteins in Table 2.4.1 would have the right alignment of amino acid side-chains to catalyze aging of the DFP label. The computer finding of the presence of aged DFP on multiple sites might simply be a coincidence of numbers.

Several major peaks in the MS/MS spectra are unassigned. Since fragmentation of OP-labeled serine and OP-labeled threonine results in loss of the OP and loss of a water molecule, the spectra were examined to see if the unassigned peaks could belong to this group. This was not the case. We found no evidence for neutral loss of OP and water from serine and threonine, and were unable to assign a structure to major peaks.

In conclusion, our evaluation of the data in Table 2.4.1 is that albumin and apolipoprotein are correctly identified as being labeled by DFP. All the other assignments are doubtful.

Evaluation of low abundance DFP-labeled peptides in Table 2.4.2. The one protein that was expected to be in this set was butyrylcholinesterase. However, butyrylcholinesterase was not found.

We have little confidence in the protein identifications in Table 2.4.2 for the following reasons:

1) Many of the peptides contain one or two missed cleavages. The computer seems to have been struggling to fit the masses, and could only do so by adding extra residues.

2) Two to four residues are labeled in 14 of the 17 peptides. This is unprecedented and therefore difficult to believe.

3) The labeled Serine residues are not in the consensus sequence GXSXG for serine hydrolases.

4) The DFP label is present as the aged monoisopropylphosphate (+122) in some cases. However, aging is not expected because aging requires a specific active site that includes amino acid side chains capable of stabilizing an aging intermediate. The proteins in Table 2.4.2 are not expected to be capable of catalyzing DFP aging.

5) Only three of the proteins in Table 2.4.2 are in the plasma proteome databank. They are ATP-binding cassette (ABCA4); synaptopodin 2, and DNA repair protein (ERCC4).

6) Many of the MS/MS spectra have a discontinuous y-ion series. This is opposite to what one normally finds for tryptic peptides.

7) Many of the MS/MS spectra contain unassigned major peaks, suggesting the presence of a mixture of parent ions, and therefore the likelihood of errors.

The best results are for testis-specific basic protein Y2. This peptide has a single DFP label. All major peaks in the MS/MS spectrum are assigned. On the negative side, the peptide contains one missed cleavage, and its MS/MS spectrum contains a discontinuous y-ion series.

Conclusion

We conclude that butyrylcholinesterase, albumin, and apolipoprotein bind DFP covalently. The data for butyrylcholinesterase come from Task 2.1 and from the literature, for albumin from Task 2.2, and for apolipoprotein from Task 2.4.

TASK 2

2.5. Identify the proteins in mouse blood that bind DFP.
reported April 20, 2007

Relation to statement of work. Results for task 2.5 are reported.

DFP-labeled mouse plasma transferrin

Summary

The purpose for analyzing proteins labeled by DFP in mouse plasma in vitro is to provide background information in preparation for studying living mice treated with DFP. In this task we focused on the plasma protein transferrin because our mass spectrometry results in Task 1 for OP labeled proteins in human plasma had identified OP-labeled transferrin. Purified mouse transferrin was treated with DFP. Tryptic peptides were analyzed by mass spectrometry. MS/MS spectra identified DFP-labeled peptides GY*YAVAVVK and KPVDQY*EDCYLAR where the OP was covalently bound to tyrosine.

Introduction

Transferrin is a glycoprotein with 697 amino acids. The mouse transferrin protein has accession # gi:17046471. Transferrin is synthesized in the liver and secreted into blood where its function is to transport iron. Transferrin was chosen for detailed study because it was identified in preliminary mass spectrometry studies as a protein labeled by OP. In this task the amino acids in transferrin that covalently bind OP were identified.

Material and Methods

Purified mouse transferrin (Sigma T0523) dissolved in phosphate buffered saline to make 25 µM transferrin, was reacted with 1 mM DFP at 37˚C for 20 hours. The disulfide bonds were reduced and alkylated with iodoacetamide. Excess reagents were removed by dialysis. The protein was digested with trypsin and the peptides separated by reverse phase chromatography on a Waters HPLC. Each HPLC fraction was analyzed in the MALDI-TOF-TOF mass spectrometer by acquiring MS and MS/MS spectra. The amino acid sequence of labeled peptides was confirmed in the QTRAP 2000 mass spectrometer. The MALDI-TOF-TOF

does not fragment peptides as well as the QTRAP 2000 mass spectrometer. Therefore, we routinely confirm our results in the QTRAP mass spectrometer.

Results and Discussion

Two tyrosine residues in mouse transferrin were labeled by DFP, Tyr 448 and 257. These same tyrosines were also labeled by FP-biotin.

Table 2.5. Mouse transferrin peptides modified by covalent binding of DFP.

DFP labeled peptide	unlabeled mass	mass + 164	labeled residue
GY*YAVAVVK	969.54	1133.54	Tyr 448
KPVDQY*EDCYLAR	1656.77	1820.77	Tyr 257

Accession # Q921I1 in SwissProt.2007.01.09 or gi:17046471 in PubMed

The same residues were labeled in apotransferrin lacking bound iron, as well as in transferrin containing two iron molecules.

In addition to binding iron, transferrin also binds two carbonate ions. Tyrosine 448 is predicted to be part of the binding site for one of the carbonate ions (ModBase UCSF, linked through SwissProt).

Knowledge of the amino acids labeled by OP provides the basic information needed for diagnosis of OP exposure. With this information one can design Selected Ion Monitoring experiments to diagnose low level OP exposure.

TASK 2

2.6. Inject mice with DFP and identify the DFP-labeled proteins in mouse blood.
reported July 20, 2007

Relation to statement of work. Results for task 2.6 are reported.

Mass spectrometry search for OP-labeled proteins after treatment of mouse with DFP

Summary

At least 12 FP-biotin labeled proteins were found in mouse plasma after a mouse was treated with FP-biotin. Our goal was to determine whether DFP also labeled a number of proteins in a mouse treated in vivo with DFP, and to identify the DFP-labeled proteins. A dose of 6.0 mg/kg DFP ip was toxic to a mouse, causing severe cholinergic signs of toxicity. The mouse was euthanized 15 min later, and the plasma proteins prepared for mass spectrometry. Tryptic peptides were analyzed by LC/MS/MS. 86 mouse proteins were identified, but none was DFP-labeled. This experiment will have to be refined to obtain a positive result.

Introduction

Mass spectrometry of OP-labeled purified proteins, and of OP-labeled plasma provides the background information for identifying OP-labeled proteins in living humans. Before we are ready to test human samples, we need to develop methods for identifying OP-labeled proteins by studying the living mouse. The number of proteins in plasma is estimated to total at least 10,000. Assuming each protein has about 20 tryptic peptides, this means one must find about 10 DFP-labeled peptides in a group of 200,000. In our first trials we used a blanket approach where we looked at every tryptic peptide in mouse plasma. This approach has not yet been successful, though we are hopeful that refinements to our search strategy will eventually succeed.

Materials and Methods

Wild type male mouse weighing 30 grams, strain 129SV, was injected intraperitoneally with 180 µl of 55.2 mg/ml (0.3 M) DFP dissolved in ethanol for a

dose of 6.0 mg/kg. The mouse was euthanized 15 min after treatment and 100 µl of plasma collected into heparinized microvet tubes (Sarstedt).

Tryptic peptides for mass spectrometry. Tryptic peptides were prepared for mass spectrometry as described in Task 2.4. In brief, mouse plasma was divided into high and low abundance proteins by passage over an antibody column that selectively binds the 12 most abundant proteins. The proteins were denatured in 8 M urea, reduced with dithiothreitol, alkylated with iodoacetamide, dialyzed to remove salts, and digested with trypsin. Tryptic peptides were separated by offline chromatography on a strong cation exchange HPLC column. HPLC fractions were pooled to give 15 samples, each containing 5 µg protein.

LC/MS/MS. 15 samples of tryptic peptides from the low abundance fraction of mouse plasma were analyzed by LC/MS/MS as described in Task 2.4 on a QTRAP 2000 mass spectrometer.

Search for DFP-modified albumin. Two adult mice were injected intraperitoneally with 3 mg/kg DFP. Plasma was collected after 1 h, 24 h, and 48 h and stored at -20 C. The plasma was analyzed for the presence of DFP-labeled albumin by acidifying 5 µl plasma with 5 µl of 1% trifluoroacetic acid and digesting with 1 µl of 0.25 mg/ml pepsin at 37˚C for 1 hour. The digest was diluted 400-fold with 0.1% trifluoroacetic acid before 1 µl was spotted on a MALDI target plate. Control samples were from mice injected with saline solution.

Results and Discussion

Toxicity. The 6.0 mg/kg dose of DFP resulted in severe whole body tremors, clonic seizures, piloerection, bugged eyes, ataxic gait, Straub tail, salivation, and splayed hind legs.

Mass spectrometry identified 86 unique proteins in mass plasma. The low abundance fraction of mouse plasma was divided into 15 groups of peptides according to their elution from a strong cationic exchange column. Each of the 15 samples was subjected to reverse phase HPLC. As each peptide eluted off the HPLC column it was electrosprayed into the mass spectrometer where masses of parent and daughter ions were obtained. Table 2.6.1 lists the 86 proteins identified in mouse plasma sorted by the number of times a peptide from that protein was identified, that is, the peptide count.

Albumin peptides were found most frequently, even though the plasma had been partially depleted of albumin. Other highly abundant plasma proteins including transferrin, alpha-1-antitrypsin, and apolipoprotein were present even though they had also been partially depleted. No esterase was in our dataset, though others have found paraoxonase, carboxylesterase, and butyrylcholinesterase in the mouse serum proteome (Hood et al., 2005).

Table 2.6.1. Mouse plasma proteome (not labeled with DFP).

Protein Name	P-value	Peptide Counts
Serum albumin precursor	7.66677E-13	393
Trf Serotransferrin precursor	5.0317E-13	91
Serpina1b Alpha-1-antitrypsin 1-2 precursor	4.45125E-14	57
Serpina1b Serpina1b protein	4.45125E-14	57
Serpina1a Alpha-1-antitrypsin 1-1 precursor	4.45125E-14	55
Serpina1c;Serpina1a Alpha-1-antitrypsin 1-3 precursor	4.45125E-14	55
Spi1-6 Alpha-1-antitrypsin 1-6 precursor	4.45125E-14	55
Apoa1 Apolipoprotein A-I precursor	3.21459E-09	48
Serpina1e Alpha-1-antitrypsin 1-5 precursor	4.45125E-14	47
Serpina3k Serine protease inhibitor A3K precursor	2.6952E-11	23
Hpxn Hemopexin precursor	8.72139E-11	19
Hbb-b1 Hemoglobin subunit beta-1	3.91519E-07	16
"Hba-a1;Hba-a2 13 days embryo liver cDNA		10
"Hba-a1;Hba-a2 hemoglobin alpha	1528.73	10
Hba-a1;Hba-a2 Hemoglobin subunit alpha	9.76107E-12	10
Mug1 Murinoglobulin-1 precursor	8.7357E-09	10
Hbb Beta-2-globin (Fragment)	3.91519E-07	9
Hbb-b2 Hemoglobin subunit beta-2	3.91519E-07	9
Serpina1d Alpha-1-antitrypsin 1-4 precursor	7.31711E-13	9
Serpina3c Serine protease inhibitor A3C precursor	2.6952E-11	9
Serpina3f Serine protease inhibitor A3F	2.6952E-11	9
Serpina3g 41 kDa protein	2.6952E-11	9
Serpina3g Serine protease inhibitor A3G	2.6952E-11	9
Serpina3k 47 kDa protein	2.6952E-11	9
Serpina3m Serine protease inhibitor A3M precursor	2.6952E-11	9
Serpina3n Serine protease inhibitor A3N precursor	2.6952E-11	9
Hbb-b1 Beta-2-globin (Fragment)	3.91519E-07	8
Hbb-b1 Hbb-b2 protein	3.91519E-07	8
"Serpina3h Activated spleen cDNA	2	7
Serpina3g Serpina3g protein	2.6952E-11	7
EG640530 similar to Murinoglobulin 1	8.7357E-09	6
Hbb-b1 Beta-maj globin gene 5' flanking region (Fragment)	8.28989E-07	6
Mus81 Crossover junction endonuclease MUS81	3.76218E-07	6
Pzp Alpha-2-macroglobulin precursor	4.58732E-09	6
C3 Isoform Long of Complement C3 precursor	6.85145E-13	5
Gc Vitamin D-binding protein precursor	1.02058E-10	5
LOC100044537 similar to Group specific component	1.02058E-10	5
Ulk4 Isoform 5 of Serine/threonine-protein kinase ULK4	1528.77	5
Ulk4 Isoform 6 of Serine/threonine-protein kinase ULK4	1528.77	5
Ulk4 similar to Serine/threonine-protein kinase ULK4	1528.77	5
Ahsg Alpha-2-HS-glycoprotein precursor	1.38153E-11	4
Mug2 Murinoglobulin-2 precursor	8.7357E-09	4
Mug4 Murinoglubulin-4 precursor	8.7357E-09	4
"4930402K13Rik Adult male testis cDNA		3
ENSMUSG00000074373;Apoa4 apolipoprotein A-IV	2.22052E-10	3
ENSMUSG00000074373;Apoa4 Apolipoprotein A-IV precursor	2.22052E-10	3
Hbb-b1 11 kDa protein	6.04792E-07	3

"Lig1 ligase I	3	2
"Serpina3f Adult male aorta and vein cDNA	2	2
Apoa2 Apolipoprotein A-II precursor	3.86415E-07	2
EG434758 Novel protein similar to reproductive homeobox 3 Rhox3	1422.67	2
Kng1 HMW kininogen-I variant	1.95161E-09	2
Kng1 Isoform HMW of Kininogen-1 precursor	1.95161E-09	2
Kng1 Isoform LMW of Kininogen-1 precursor	1.95161E-09	2
Kng1 Kng1 protein	1.95161E-09	2
Lig1 DNA ligase	4.36079E-08	2
LOC329984 Novel protein similar to the preferentially expressed antigen in melanoma like (Pramel) family	4.89042E-08	2
Prdm15 PR domain containing 15	2213.08	2
RP23-33P17.2 Novel protein similar to the preferentially expressed antigen in melanoma like (Pramel) family	4.89042E-08	2
Serpina3g Spi2 proteinase inhibitor (Fragment)	1.73506E-07	2
Sfrp2 Secreted frizzled-related protein 2 precursor	9.96846E-08	2
"Fga fibrinogen	1491.7	1
"Fgg Adult male liver tumor cDNA		1
"Heatr5b 13 days embryo heart cDNA		1
"Mthfd1l Osteoclast-like cell cDNA		1
"Ptprf protein tyrosine phosphatase		1
"Rab26;Traf7 2 days neonate thymus thymic cells cDNA		1
- 6 kDa protein	4.32834E-06	1
- Putative uncharacterized protein Vps13a 3' variant II (Fragment)	3.50103E-06	1
C3 Isoform Short of Complement C3 precursor (Fragment)	2.21717E-07	1
Dpp3 Dipeptidyl-peptidase 3	2.14785E-06	1
EG434758 hypothetical protein	1422.67	1
ENSMUSG00000071772 Testis expressed homeobox 2	1422.67	1
Hbb-y;LOC100044141 Hemoglobin subunit epsilon-Y2	4.32834E-06	1
Heatr5b HEAT repeat containing 5B	2.7539E-06	1
Itgav Integrin alpha-V precursor	5.34615E-06	1
LOC100044141 similar to beta-globin isoform 2	4.32834E-06	1
LOC100044312;OTTMUSG00000017155 Novel protein similar to reproductive homeobox 3 Rhox3	1422.67	1
LOC546294 similar to reproductive homeobox on X chromosome 3 isoform 2	1422.67	1
Rab26 66 kDa protein	3.4913E-05	1
Rhox3 Reproductive homeobox on X chromosome 3	1422.67	1
Scgb1a1 Uteroglobin precursor	6.0848E-06	1
Traf7 E3 ubiquitin-protein ligase TRAF7	3.4913E-05	1
Vps13a Isoform 1 of Vacuolar protein sorting-associated protein 13A	3.50103E-06	1
Vps13a Isoform 2 of Vacuolar protein sorting-associated protein 13A	3.50103E-06	1

Mass spectrometry search for DFP-labeled proteins. Out of 10,000 peptides analyzed, only three had a probability score that made it likely the protein was labeled with DFP. The candidate proteins are listed in Table 2.6.2.

Table 2.6.2. DFP-labeled peptides in a mouse treated with DFP.

sequence	protein name	accession #	OMMSA score	labeled residue	DFP
LETNTSPHANK	breast cancer type 2 susceptibility protein BRCA2	AAC23702	7.431e-001	Ser 6	+164
MSSELINVSGR	retinitis pigmentosa 1 homolog Rp1h	NP_035413	1.691e-001	Ser 2 Ser 3 Ser 9	+164 +164 +164
STVTIGLVQALTAHLK	methylene tetrahydrofolate dehydrogenase	NP_758512	4.361e-001	Ser 1 Thr 2	+164 +164

The search engine did not find carboxylesterase, butyrylcholinesterase, or acetylcholinesterase, the proteins that we know bind DFP. The three peptides in Table 2.6.2 are from proteins that are not known to bind OP covalently. The assignments are weak because of a) discontinuous y-ion and discontinuous b-ion series in the MS/MS spectra, b) the presence of 1 or more major unassigned peaks in the MS/MS spectra, and c) more than one DFP labeled serine or threonine in one peptide. The best MS/MS data are for peptide LETNTSPHANK because only one major peak is unassigned, and the peptide binds only one DFP molecule.

DFP-labeled albumin. Table 2.6.3 shows the peptide sequences potentially identified as DFP-labeled mouse albumin peptides. MALDI-TOF showed small peaks at 601 and 1853 in DFP-treated mouse samples, but not in untreated control samples. The intensity of the peaks was about 5% of the intensity of the unlabeled parent ion. No peaks corresponding to aged DFP-mouse albumin were seen. The data are not yet convincing. We need MS/MS spectra of the DFP-peptides before we are certain that these peaks represent DFP-labeled mouse albumin.

Table 2.6.3. Search for DFP-albumin peptides in a mouse treated with DFP.

sequence	m/z, no label	+164 DFP not aged	+ 122 DFP aged
VRY*	437.5	601.5	559.5
VRY*TQKAPQVSTPTL	1689.9	1853.9	1811.9

Accession # 3647327. The asterisk indicates the Tyrosine to which DFP is covalently bound.

Diagnosis of OP exposure. Diagnosis of OP exposure by mass spectrometry of plasma proteins is a new area of research. The literature contains only three

examples of successful strategies for identification of OP exposure using plasma as the starting material and mass spectrometry of proteins as the tool. A paper from the U.K. Defence Science and Technology Laboratory in Porton Down reported finding tyrosine adducts of sarin, soman, cyclosarin and tabun in plasma of guinea pigs treated with these nerve agents (Williams et al., 2007). The plasma was digested with pronase, enriched by solid phase extraction on a C18 or C8 cartridge, and analyzed by LC/MS/MS on a Finnigan TSQ Quantum mass spectrometer. The identity of the soman-labeled protein was not known, though it was suggested to be albumin.

A paper from The Netherlands Division of Chemical and Biological Protection, TNO Prins Maurits Laboratory in Rijswijk reported finding sarin-labeled butyrylcholinesterase in plasma from a victim of the Tokyo subway attack by Aum Shinrikyo cult members (Fidder et al., 2002). The BChE from 0.5 ml plasma was enriched by procainamide affinity chromatography, digested with pepsin, and analyzed by LC/MS/MS. The sarin-labeled active site peptide of butyrylcholinesterase was positively identified.

A third paper is from our laboratory (Peeples et al., 2005) in the United States. Mice treated with FP-biotin were found to have FP-biotin-labeled albumin in all tissues.

The long range goal of this work is to develop mass spectrometry methods for analyzing OP exposure in humans. We have succeeded in this goal, though our success is not reported in this Final Report because analysis of human plasma samples was not in our set of Tasks. However, we have devised a method to analyze OP exposure in humans by mass spectrometry and have submitted a paper for publication on this topic. Our paper is entitled

Fast Affinity Purification Coupled with Mass Spectrometry for Identifying Organophosphate Labeled Plasma Butyrylcholinesterase

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submitted to Chemical Biological Interactions, October 2007.

KEY RESEARCH ACCOMPLISHMENTS

- Three new biomarkers of exposure to OP have been identified in human blood: albumin, transferrin, and apolipoprotein.
- Albumin, transferrin, and apolipoprotein bind OP covalently to tyrosine. This finding is contrary to the expectation that OP bind exclusively to serine.
- A new mass spectrometry assay has been developed to measure OP exposure in blood. The assay uses MALDI-TOF to measure the mass of the albumin active site peptide after covalent OP binding. This identifies the fact of OP exposure and also identifies the OP.
- A method has been developed to diagnose OP exposure in humans by analyzing plasma samples for OP-labeled butyrylcholinesterase.

REPORTABLE OUTCOMES

- Li B, Schopfer LM, Hinrichs SH, Masson P, Lockridge O. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr411. Anal Biochem. 2007 Feb 15;361(2):263-72.
- Poster at IX International Meeting on Cholinesterases in Suzhou, China 6-10 May 2007. MALDI-TOF assay for organophosphate binding to Tyr 411 of human albumin. Li B, Schopfer LM, Duysen EG, Hinrichs SH, Masson P, Lockridge O.
- Li H, Tong L, Schopfer LM, Masson P, Lockridge O (2008) Fast Affinity Purification Coupled with Mass Spectrometry for Identifying Organophosphate Labeled Plasma Butyrylcholinesterase. submitted to *Chemical Biological Interactions*, October 2007.
- Funding applied for and received based on work supported by this award:

U01 NS058056 from NIH. Title: Mass spectrometry in clinical diagnosis of nerve agent exposure. 27 Sept 2006 to 31 May 2009. Principal Investigator: O. Lockridge

PRO64275 from the Department of Defense. Title: Mass spectrometry to identify new biomarkers of nerve agent exposure. 1 April 2007 to 31 March 2009. Principal Investigator: O. Lockridge

CONCLUSION

Our mass spectrometry results contradict the dogma that serine esterases and serine proteases are the only class of proteins modified by exposure to OP. We have found that tyrosine 411 of human albumin, tyrosine 238 of human transferrin, and tyrosine 172 of apolipoprotein are covalently modified by OP. The significance of this finding is in diagnosis of OP exposure. It now becomes possible to look for several proteins in human blood, in addition to butyrylcholinesterase and acetylcholinesterase, for evidence of OP exposure. The method we have developed to date for diagnosis of OP exposure is a mass spectrometry method. However, it now becomes possible to develop a simple antibody based assay to use in the field. The information we obtained from mass spectrometry allows us to propose a natural epitope on the surface of human albumin as the OP antigen. A dipstick type assay for OP exposure is planned as

the ultimate result of this work.

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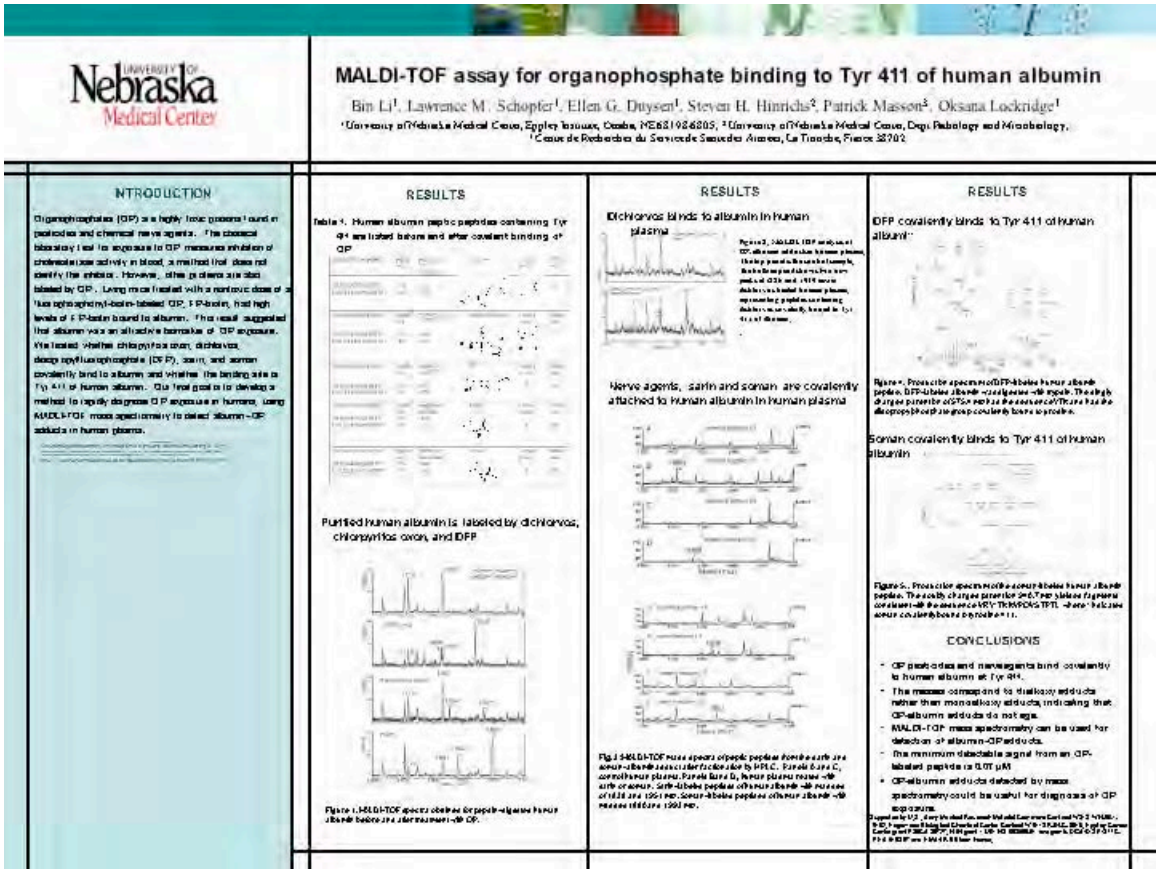

MALDI-TOF assay for organophosphate binding to Tyr 411 of human albumin

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INTRODUCTION

RESULTS

Purified human albumin is labeled by dichlorvos, chlorpyrifos oxon, and DFP

RESULTS

Dichlorvos binds to albumin in human plasma

Nerve agents, sarin and soman, are covalently attached to human albumin in human plasma

RESULTS

DFP covalently binds to Tyr 411 of human albumin

Soman covalently binds to Tyr 411 of human albumin

CONCLUSIONS

- OP pesticides and nerve agents bind covalently to human albumin at Tyr 411.
- The masses correspond to dialkoxy adducts rather than monoalkoxy adducts, indicating that OP-albumin adducts do not age.
- MALDI-TOF mass spectrometry can be used for detection of albumin-OP adducts.
- The minimum detectable signal from an OP-labeled peptide is 0.01 µM.
- OP-albumin adducts detected by mass spectrometry could be useful for diagnosis of OP exposure.

Poster presented by Bin Li at the IX International Meeting on Cholinesterases in Suzhou, China 6-10 May 2007

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr411

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Abstract

Our goal was to determine whether chlorpyrifos oxon, dichlorvos, diisopropylfluorophosphate (DFP), and sarin covalently bind to human albumin. Human albumin or plasma was treated with organophosphorus (OP) agent at alkaline pH, digested with pepsin at pH 2.3, and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Two singly charged peaks *m*/*z* 1718 and 1831, corresponding to the unlabeled peptide fragments containing the active site Tyr411 residue, were detected in all samples. The sequences of the two peptides were VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL. The peptide–OP adducts of these peptides were also found. They had masses of 1854 and 1967 for chlorpyrifos oxon, 1825 and 1938 for dichlorvos, 1881 and 1994 for DFP, and 1838 and 1938 for sarin; these masses fit a mechanism whereby OP bound covalently to Tyr411. The binding of DFP to Tyr411 of human albumin was confirmed by electrospray tandem mass spectrometry and analysis of product ions. None of the OP–albumin adducts lost an alkoxy group, leading to the conclusion that aging did not occur. Our results show that OP pesticides and nerve agents bind covalently to human albumin at Tyr411. The presence of Tyr411 on an exposed surface of albumin suggests that an antibody response could be generated against OP–albumin adducts.

Keywords: Biomarker organophosphate exposure; Pepsin; Sarin; Soman; Dichlorvos; Diisopropylfluorophosphate; Chlorpyrifos oxon; Nerve agents; Pesticides

The acute toxicity of organophosphorus (OP)[1] toxicants is known to be due to inhibition of acetylcholinesterase. However, other proteins also bind OP, although their role in toxicity is less defined [1]. Albumin is a potential new biomarker of OP exposure. Mice treated with a nontoxic dose of a biotinylated nerve agent analog, FP-biotin (10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide), had 1000 times more FP-biotinylated albumin than FP-biotinylated butyrylcholinesterase in their blood [2].

Albumin has been shown to covalently bind radiolabeled diisopropylfluorophosphate (DFP). Human albumin incorporated 1 mol DFP per mole of albumin when 20 to 70 μM albumin was incubated with a sevenfold molar excess of DFP at pH 8.0 for 2 h at 23 °C [3,4]. Bovine albumin also incorporated 1 mol DFP per mole of albumin [5]. The site of covalent binding of DFP to human albumin was identified by amino acid sequencing. The labeled peptide

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[1] *Abbreviations used:* OP, organophosphorus; FP-biotin, 10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide; DFP, diisopropylfluorophosphate; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DHBA, 2,5-dihydroxybenzoic acid; CHCA, α-cyano 4-hydoxycinnamic acid; ACTH, adrenocorticotropic hormone; MS/MS, tandem mass spectra; LC–MS, liquid chromatography–mass spectrometry; GC–MS, gas chromatography–mass spectrometry.

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had the sequence ArgTyrThrLys with DFP bound to Tyr [6]. Later, when the complete amino acid sequence of human albumin was known, the active site tyrosine was identified as Tyr411 (Tyr435 when residue 1 is Met of the signal peptide). Mass spectrometry (MS) identified Tyr410 of bovine albumin (equivalent to Tyr411 of human albumin) as the covalent binding site for FP-biotin [7]. The nerve agents soman and sarin were shown to bind covalently to human albumin on tyrosine [8,9] and to be released by treatment with potassium fluoride [9].

Albumin has also been demonstrated to be an OP hydrolase, hydrolyzing chlorpyrifos oxon, *O*-hexyl *O*-2,5-dichlorophenylphosphoramidate, and paraoxon at measurable rates [10–13]. The apparent K_m of bovine albumin is 0.41 mM for chlorpyrifos oxon and 1.85 mM for paraoxon [12], and the apparent K_m of human albumin is 3.6 mM for DFP [3]. Despite this seemingly consistent body of results, some issues have been raised regarding the reaction of OP with albumin. It has been questioned whether the observed OP hydrolase activity was associated with the albumin molecule itself or with minor phosphotriesterase contaminants in the albumin preparation [10]. In addition, the possibility has been raised that DFP binds to one site in albumin but that other OP toxicants bind to a different site [12,14].

Our goal was to determine whether Tyr411 of human albumin was the site for covalent attachment of a variety of OP toxicants. For this purpose we developed a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS assay applicable to purified human albumin and to human plasma.

Materials and methods

Materials

Purified human serum albumin, essentially fatty acid free, was obtained from Fluka via Sigma (Cat. No. 05418, St. Louis, MO, USA). Pepsin from porcine gastric mucosa was obtained from Sigma (Cat. No. P6887). Modified trypsin, sequencing grade, was purchased from Promega (Cat. No. V5113, Madison, WI, USA). DFP was obtained from Sigma (Cat. No. D0879). Dichlorvos and chlorpyrifos oxon were purchased from Chem Services (Cat Nos. PS-89 and MET-674B, West Chester, PA, USA). Sarin-treated human plasma was a gift from Patrick Masson. Acetonitrile high-performance liquid chromatography (HPLC)-grade 99% ACROS, was purchased from Fisher Scientific (Cat. No. 61001-0040, Pittsburgh, PA, USA). Trifluoroacetic acid (TFA), sequencing grade, was purchased from Beckman Instruments (Cat. No. 290203, Palo Alto, CA, USA). 2,5-Dihydroxybenzoic acid (DHBA) matrix was purchased from Applied Biosystems (Foster City, CA, USA). α-Cyano 4-hydroxycinnamic acid (CHCA, Cat. No. 70990, Sigma) was recrystallized before use. Calibration standards for MALDI-TOF were obtained from New England Biolabs (Cat. No. P7720S, Beverly, MA, USA) and included angiotensin 1 (1297.51 amu), adrenocorticotropic hormone (ACTH) (7–38) (3660.19 amu), and ACTH (18–39) (2466.73 amu). Double distilled water was prepared in-house and was autoclaved.

Sample preparation for DFP-, dichlorvos-, and chlorpyrifos oxon-treated samples

Fatty acid-free human albumin at a concentration of 10 mg/ml, which is 150 μM, was dissolved in 25 mM ammonium bicarbonate (pH 8.6) and treated with an equimolar concentration of OP for 24 h at 37 °C. The pH of 1000 μl reaction mixture was reduced to 2.3 by the addition of 500 μl of 1% TFA. Pepsin was dissolved in 10 mM HCl to make 1 mg/ml and was stored at −80 °C. The albumin was digested with pepsin (1:250 ratio) for 2 h at 37 °C and was diluted to 1 pmol/μl with 0.1% TFA.

A 200-μl aliquot of human plasma was treated with 6.85 μl of 20 mM OP (660 μM final OP concentration) for 24 h at 37 °C. The pH was adjusted to 2.3 to 2.5 by the addition of 200-μl of 1% TFA. Proteins were digested with 50 μl of 1 mg/ml pepsin for 2 h at 37 °C. Before spotting the digest on the target plate, a 10-μl aliquot of the digest was diluted with 390 μl of 0.1% TFA so that the final plasma dilution was 1000-fold.

MALDI-TOF

A 1-μl aliquot of diluted peptic digest was applied to a stainless-steel target plate, air-dried, and overlaid with 1 μl of 2,5-dihydroxybenzoic acid matrix. The CHCA matrix gave similar results. Mass spectra were acquired with the Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems/MDS Sciex) in linear positive ion mode, 20,000 V accelerating voltage, 94% grid voltage, 0.1% guide wire, 350 ns extraction delay time, and automated laser intensity adjustment from 1000 to 1600. The instrument was calibrated with a peptide calibration mixture from New England Biolabs. Mass accuracy for each standard was within 0.05% of the corresponding average molecular weight. Spectra were acquired in automatic mode by examining signals from random spots on a target. The signals from the first 10 spots that met the acceptance criteria were summed into one final profile mass spectrum The acceptance criteria were signal intensities between 1000 and 55,000 counts with signal/noise ratios of 10 or greater and minimum resolution of 50. The final spectrum was the average of 1000 shots.

The MS-Digest program from the UCSF Mass Spectrometry Facility was used to calculate the masses of the peptic peptides expected from digests of human serum albumin.

Quadrupole mass spectrometer

Tandem mass spectra (MS/MS) were acquired on a Q-Trap 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex) with a nano electro-

spray ionization source. DFP-labeled albumin digested with trypsin was infused into the mass spectrometer via a fused silica emitter (360 μm o.d., 20 μm i.d., 10 μm taper, New Objective, Woburn, MA, USA) using a Harvard syringe pump to drive a 100-μl Hamilton syringe equipped with an inline 0.25-μ filter at a flow rate of 1 μl/min. Samples were prepared in 50% acetonitrile and 0.1% formic acid. Positive ion spectra were obtained. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with collision energy of 40 ± 5 V and nitrogen gas pressure of 4×10^{-5} Torr. The final enhanced product ion scan was the average of 212 scans.

Sample preparation of sarin-treated plasma

Human plasma (100 μl) was treated with 600 μM sarin and stored at ambient temperature for 3 days. This concentration of sarin is equimolar with the concentration of albumin in plasma. Then 10 μl was digested with 0.5 μg of pepsin at 37 °C for 2 h at pH 2.3, and the peptides were separated by HPLC on a Waters 625 LC system. A C18 reverse-phase column (Prodigy 5-μ ODS(2), 100 × 4.6 mm, 5 μ, 00 D-3300-E0, Phenomenex, Torrance, CA, USA) was used to trap the peptides from the digest, which were then eluted with a 40-min gradient starting with 85% buffer A (0.1% TFA in water) and 15% buffer B (acetonitrile containing 0.07% TFA) and ending with 65% buffer A and 35% buffer B. Then 1-ml fractions were reduced in volume to 200 μl in a vacuum centrifuge, and 1 μl was analyzed by MALDI-TOF. A control plasma sample was treated identically except that it was incubated with 3.5% isopropanol rather than with sarin.

Results

Reaction of pure human albumin with OP

The assay was developed with pure human albumin and later tested with human plasma. Fatty acid-free albumin was used because fatty acids and OP bind to the same albumin domain and therefore fatty acids could block the binding of OP [3,15]. The covalent attachment site for human albumin, Tyr411, is located near the surface of the albumin molecule, where it is accessible to proteases. Digestion with trypsin at pH 8.6 or with pepsin at pH 2.0 to 2.5 released peptides of the expected masses without the need to denature or to reduce and alkylate the disulfide bonds of albumin. Peptides containing Tyr411 had the sequence YTK (*m*/*z* 411, singly charged mass) when the protease was trypsin and had the sequences VRYTKKVPQVSTPTL (*m*/*z* 1718) and LVRYTKKVPQVSTPTL (*m*/*z* 1831) when the protease was pepsin. Pepsin routinely missed one cleavage in our experiments.

The tryptic YTK peptide (*m*/*z* 411) and the dichlorvos, chlorpyrifos oxon, and DFP adducts had masses that overlapped with matrix peaks, making them difficult to detect by MALDI–MS. Furthermore, the YTK peptide and YTK–OP adducts did not seem to ionize when irradiated by the nitrogen laser in the Voyager DE-PRO, although they did ionize in the electrospray source of the Q-Trap mass spectrometer. In contrast, the larger peptides produced by digestion of albumin with pepsin separated well from matrix and ionized to give good signals in the Voyager DE-PRO. Therefore, samples intended for analysis by MALDI-TOF were digested with pepsin rather than with trypsin.

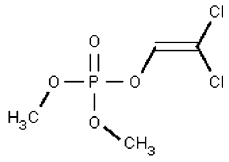
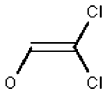
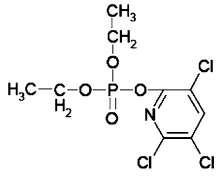
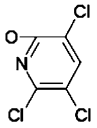
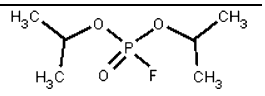
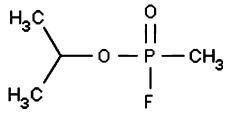
Table 1 lists the expected peptic peptide masses before and after covalent binding of dichlorvos, chlorpyrifos oxon, DFP, and sarin to Tyr411 of human albumin. The leaving group in Table 1 is that portion of the OP molecule that detaches from the OP on covalent binding of the OP to protein. The mass of the leaving group is absent from the final adduct. The added OP mass comes from the phosphorus atom, the two phosphorus ligands, and the phosphoryl oxygen atom, less one hydrogen.

Fig. 1 shows the MALDI-TOF spectra obtained for pepsin-digested human albumin before and after treatment with OP. The top panel shows masses at *m*/*z* 1718 and 1831, which are consistent with the peptides from unlabeled albumin that contain Tyr411. Additional albumin peptides are also present, but they do not contain Tyr411 and therefore are not discussed. The dichlorvos panel shows peaks at *m*/*z* 1718 and 1831 as well as two new peaks at *m*/*z* 1826 and 1939. The two new peaks have the expected sizes for the dimethoxyphosphate adducts of the *m*/*z* 1718 and 1831 peptides. The amount of labeled albumin estimated from the relative peak areas is 65%. The chlorpyrifos oxon panel shows two new peaks at *m*/*z* 1854 and 1967 for the diethoxyphosphate adducts. Approximately 30% of the albumin is labeled. The DFP panel shows two new peaks at *m*/*z* 1882 and 1995 for the diisopropoxyphosphate adducts. Approximately 70% of the albumin is labeled. Because the MALDI conditions disrupt noncovalent interactions, the OP–peptide adducts must be covalently formed. These results support the conclusion that human albumin is labeled by dichlorvos, chlorpyrifos oxon, and DFP and are consistent with the site for covalent attachment being Tyr411. The masses correspond to the dialkoxy adducts rather than to the monoalkoxy adducts. Masses for monoalkoxy adducts were not found, supporting the conclusion that OP–albumin adducts do not age.

Saturating the albumin binding sites

Unlabeled *m*/*z* 1718 and 1831 peptides always were present when the concentration of OP was the same as the concentration of albumin. However, when the dichlorvos or DFP concentration was 40-fold higher than the albumin concentration, all of the Tyr411 sites were occupied and no unlabeled peptides of *m*/*z* 1718 and 1831 were detected. OP labeling reactions with albumin were performed at pH 8.5 because labeling occurs at high pH but is decreased markedly at neutral pH [3,5].

Table 1
Pepsin digested human albumin

Human albumin peptide	Peptide *m/z*	Peptide *m/z* after dichlorvos	Dichlorvos	Leaving group	Added mass
VRYTKKVPQVSTPTL	1718	1826			108
LVRYTKKVPQVSTPTL	1831	1939			
		Peptide *m/z* after chlorpyrifos oxon	Chlorpyrifos oxon		
VRYTKKVPQVSTPTL	1718	1854			136
LVRYTKKVPQVSTPTL	1831	1967			
		Peptide *m/z* after DFP	DFP		
VRYTKKVPQVSTPTL	1718	1882		F	164
LVRYTKKVPQVSTPTL	1831	1995			
		Peptide *m/z* after sarin	Sarin		
VRYTKKVPQVSTPTL	1718	1838		F	120
LVRYTKKVPQVSTPTL	1831	1951			

Note. The $[M+H]^{+1}$ masses of peptic peptides containing Tyr411 are listed before and after covalent binding of OP. Tyr411 is underlined. The accession number for human albumin is GI:28592 where Tyr411 is Tyr435 because numbering begins with the signal peptide.

Limit of detection

It was essential to dilute the albumin and plasma digests before applying the sample on the target plate. Undiluted samples did not show the desired peptides due to ion suppression and charge competition effects. The limit of detection of OP-labeled peptide was determined from dilutions of peptides in which all of the Tyr411 sites were occupied. The diluent was 0.1% TFA in water. Peaks of interest were detected after 100-, 1000-, 3000-, and 9000-fold dilutions of a sample whose starting concentration was 600 μM (40 mg/ml) albumin. The signal/noise ratio for the 1:9000 diluted sample was 3:1. At 18,000-fold dilution, the peak height was only twofold above the noise. Thus, the minimum detectable signal from an OP-labeled peptide occurred at 0.07 pmol/μl.

Missed cleavage

We hoped to increase the sensitivity of the assay by changing digestion conditions so that pepsin would consistently cleave Leu from the N terminus of the active site peptide. If there were no missed cleavage, the area of the 1721 peak would increase relative to the background noise, thereby increasing the sensitivity of the assay. We increased the pepsin/albumin ratio to 1:25, lowered the pH in increments to 1.5, and increased the digestion time to 4 h. A pepsin/albumin ratio of 1:25 at pH 1.8 incubated for 2.5 h at 37 °C did eliminate the 1831 peptide but did not increase the signal for 1721.

Detection of dichlorvos bound to albumin in human plasma

The concentration of albumin in human plasma is approximately 40 to 50 mg/ml (600–770 μM). No other protein in plasma is present at such a high concentration. This overwhelming concentration of albumin in plasma suggested that it might be possible to detect OP–albumin adducts without separating albumin from other proteins in plasma. This was tested by incubating an aliquot of human plasma with a concentration of dichlorvos equimolar to the albumin concentration in plasma. The pH was not adjusted, and no buffer was added for the reaction with dichlorvos. Then the plasma was digested with pepsin at pH 2.3 and diluted 600-fold with 0.1% TFA to yield an albumin peptide concentration of approximately 1 pmol/μl. A 1-μl aliquot of the diluted digest was applied to the MALDI target. The control sample was human plasma treated in parallel with everything except dichlorvos. Fig. 2 shows that albumin peptides at *m/z* 1718 and 1831 stand out, despite the presence of normal plasma components, and that the OP-labeled form of these peptides at *m/z* 1826 and

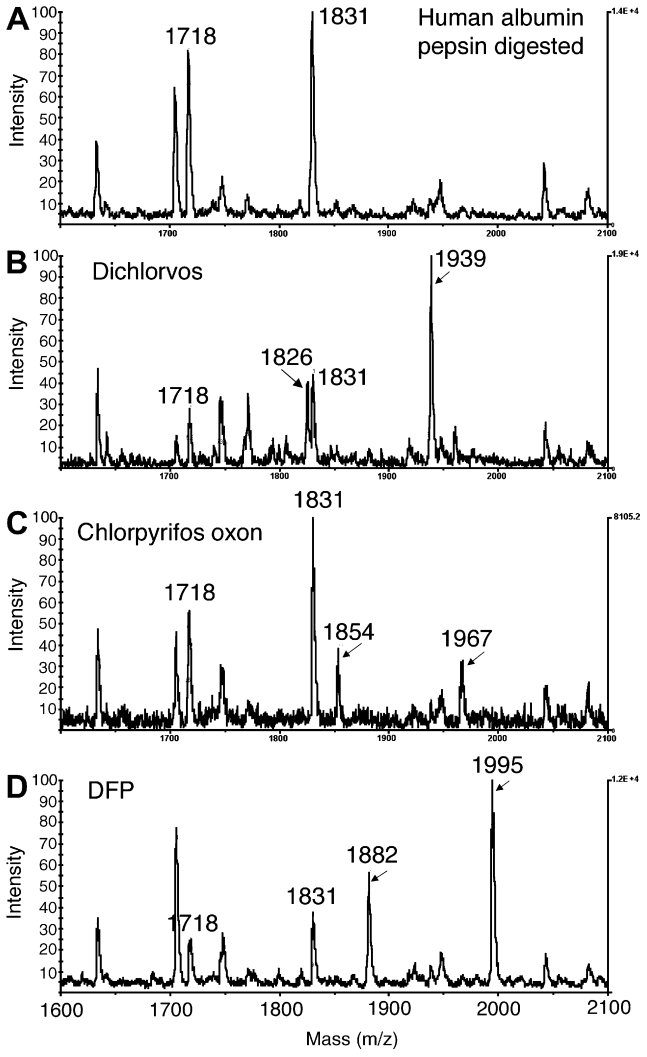


Fig. 1. MALDI-TOF analysis of human albumin–OP adducts. (A) Digestion of human albumin with pepsin at pH 2.3 yields two peptides containing Tyr411 whose average mass/charge ratios are 1718 and 1831 (singly protonated). (B) Incubation of human albumin with dichlorvos in ammonium bicarbonate (pH 8.5), followed by digestion with pepsin (pH 2.3), yields dimethoxyphosphate adducts of *m/z* 1826 and 1939. (C) Incubation with chlorpyrifos oxon yields diethoxyphosphate adducts of *m/z* 1854 and 1967. (D) Incubation with DFP yields diisopropoxyphosphate adducts of *m/z* 1882 and 1995. Samples were diluted to 1 pmol/μl before plating 1 μl on the MALDI target with 2,5-dihydroxybenzoic acid matrix.

1939 can be detected by MALDI–TOF. We conclude that human plasma can be assayed for OP bound to albumin. The 1826 and 1939 masses are 108 amu larger than their unlabeled counterparts, identifying the OP as a dimethoxy OP and thus classifying it as a pesticide rather than as a nerve agent. For forensic purposes, it is valuable to know whether the OP is a pesticide or a nerve agent.

Detection of sarin bound to albumin in human plasma

MALDI-TOF analysis of peptic digests of human plasma labeled by reaction with 600 μM sarin did not show the sarin-labeled peptides after simple dilution. We suspected that the absence of signal was due to ion suppression. Therefore, the digests were fractionated by reverse-phase chromatography prior to mass spectral analysis, as shown in Fig. 3. Fractions were collected at 1-min intervals. Each HPLC fraction was analyzed by MALDI-TOF. Peptides of interest eluted between 8 and 16 min. The unlabeled active site peptides of albumin, of masses 1718 and 1831, eluted at 8 to 10 min. The sarin-labeled peptides of masses 1838 and 1951 eluted at 14 to 16 min (Fig. 4, fractions 14 and 16). The peptides of interest separated from a large peak of UV absorbing material in the HPLC. Elimination of this material would be expected to reduce ion suppression, thereby accounting for the appearance of the sarin-labeled peptide signals in MALDI-TOF analysis.

Fig. 4 shows the MALDI-TOF spectra for the two sarin-labeled peptides of human albumin with masses of 1838 and 1951. These masses are consistent with the 1718 and 1831 albumin peptides to which 120 amu from sarin has been added. The fact that the sarin–peptide complex survived the MALDI conditions indicates that sarin has made a covalent complex. These results show that sarin covalently binds to human albumin on Tyr411. They also show that binding can be detected in plasma and that the sarin adduct of albumin does not lose an alkoxy group during storage for 3 days at room temperature.

Confirmation of DFP binding to Tyr411 of the YTK peptide

DFP-labeled human albumin was digested with trypsin, and the digest was infused into the Q-Trap mass spectrometer. The enhanced mass spectrum showed a peak at *m/z* 575.4, which is consistent with the singly charged YTK peptide covalently bound to DFP ($[M+H]^{+1} = 411$ amu for the YTK peptide plus 164 amu added mass from DFP [Table 1]). This peptide was subjected to collision-induced dissociation with nitrogen as the collision gas. The resulting enhanced product ion spectrum yielded amino acid sequence information consistent with the sequence YTK where DFP is covalently bound to tyrosine (Fig. 5). A mass was found at 147.2 amu, indicative of the C-terminal lysine from a *y*-series. This was followed by masses at 248.3, for the Thr-Lys dipeptide, and at 575.4, for the Tyr*ThrLys tripeptide, including the N terminus, from a *y*-series (where Tyr* represents the diisopropylphospho adduct of tyrosine). No relevant signals were found at higher masses. No evidence for the diisopropylphospho adduct of threonine was found.

Furthermore, convincing evidence for covalent binding of DFP to Tyr411 comes from the presence of six masses, all of which are consistent with various fragments of DFP attached to tyrosine alone or in conjunction with the YTK peptide. The structures of these six ions are shown in Fig. 5. As mentioned earlier, the ion at 575.4 amu is consistent with the singly protonated YTK peptide plus the added mass from covalent attachment of DFP. Neutral loss of 42 amu yields the 533.4-amu ion. Loss of 42 amu is predicted for β-elimination of propylene from the diisopropylphosphate adduct. This β-elimination-type reaction, also referred to as a McLafferty rearrangement [16,17], is a facile

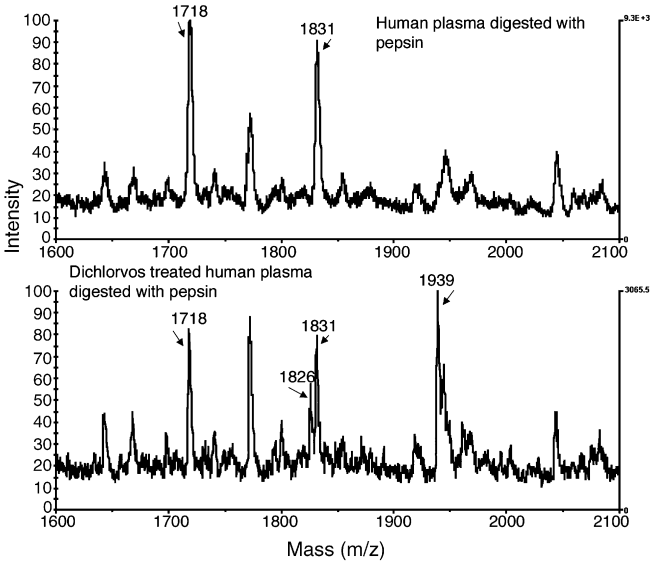


Fig. 2. MALDI-TOF analysis of OP–albumin adducts in human plasma. The top panel is the control sample. Human plasma digested with pepsin shows the Tyr411 containing albumin peptides of *m/z* 1718 and 1831. The bottom panel shows two new peaks at *m/z* 1826 and 1939 in dichlorvos-treated human plasma, representing peptides containing dichlorvos covalently bound to Tyr411 of albumin.

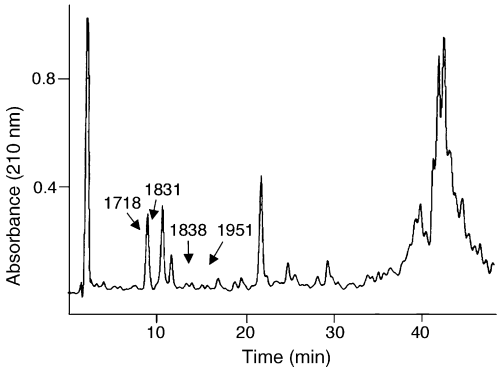


Fig. 3. HPLC trace. A peptic digest of human plasma (10 μl) that had been treated with sarin was subjected to reverse-phase chromatography. Unlabeled active site albumin peptides of masses 1718 and 1831 separated from sarin-labeled albumin peptides of masses 1838 and 1951 and from a large peak of UV absorbing material. The marked peaks contain a mixture of peptides; therefore, the relative UV intensities do not represent the relative amounts of labeled and unlabeled albumin.

reaction commonly seen during collision-induced dissociation of phosphopeptides [18]. A second neutral loss of 42 amu yields the 491.3-amu ion; this mass is consistent with a phosphorylated YTK peptide. In theory, all three of these masses are consistent with DFP adducts of either tyrosine or threonine. However, the mass at 244.2 amu is characteristic of an N-terminal phosphotyrosine, *b*-series aziridone ion, and the 226.2-amu ion is consistent with its dehydration product [19]. In addition, the mass at 216.2 amu is characteristic of a phosphotyrosine immonium ion [20]. Furthermore, no indication of phosphorylated or organophosphorylated threonine was found. These results prove that DFP covalently binds to Tyr411 of human albumin.

No evidence for any form of the dephosphorylated YTK peptide was found. This probably reflects the relative difficulty of releasing OP from tyrosine compared with the other

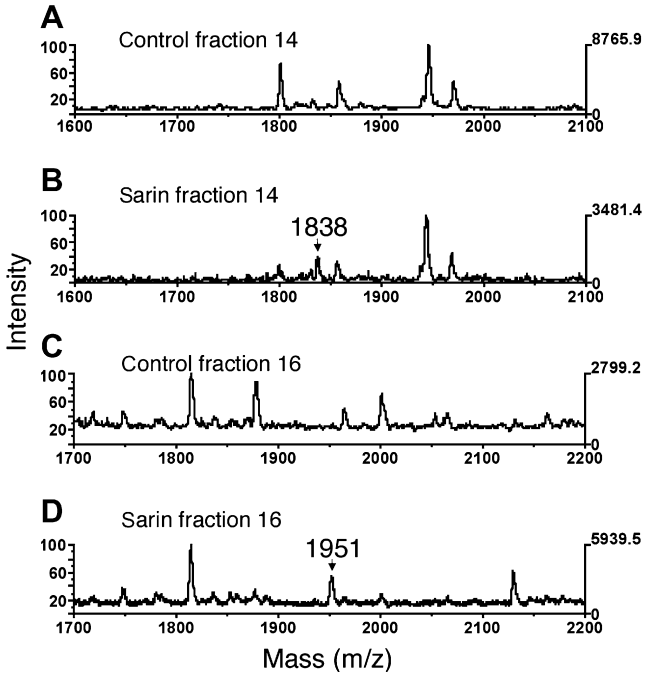


Fig. 4. MALDI-TOF analysis of sarin covalently attached to human albumin. (A and C) Control human plasma digested with pepsin and fractionated by HPLC but not treated with sarin. (B and D) Human plasma treated with sarin before digestion with pepsin and fractionation by HPLC. Fractions were collected at 1-min intervals. Sarin-labeled albumin peptides of 1838 and 1951 amu include 120 amu from sarin.

fragmentation pathways available to the diisopropylphospho–YTK peptide. Facile dephosphorylation of phosphotyrosine via a β-elimination-type mechanism is not available because tyrosine does not afford a suitable environment. β-Elimination would require the shift of a proton from the β-carbon of the leaving group to the phosphate oxygen, with concomitant formation of a double bond between the α- and

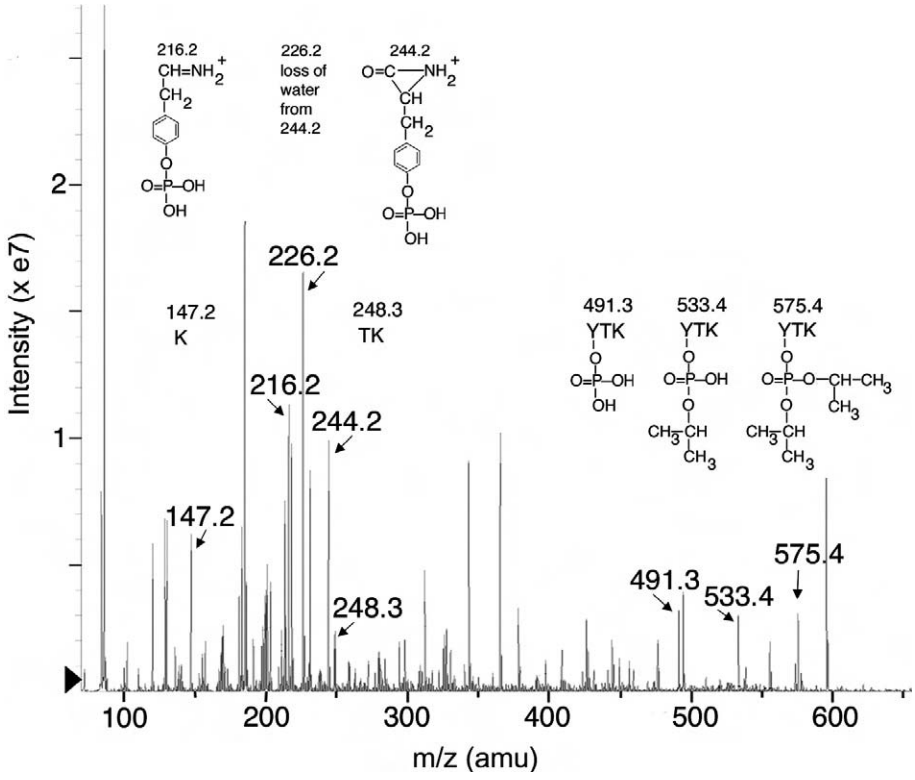


Fig. 5. Product ion spectrum of DFP-labeled human albumin peptide 575.4. DFP-labeled albumin was digested with trypsin and infused into the Q-Trap mass spectrometer. The singly charged parent ion of *m/z* 575.4 has the sequence YTK and has the diisopropylphosphate group covalently bound to tyrosine. Fragment masses and their corresponding structures are shown. In addition to information on the DFP–YTK peptide, fragments from a second albumin peptide (LVNEVTEFAK, doubly protonated) can be extracted from the fragmentation information.

β-carbons of the leaving group. For phosphotyrosine, the β-proton of the leaving tyrosine is aromatic and therefore not readily released; furthermore, formation of the double bond requires introduction of a triple bond into the aromatic ring of tyrosine—another difficult operation. Although phosphate can be released from tyrosine during collision-induced dissociation, it seems to require the presence of hydroxyl moieties on the phosphate. Even then, the yield of dephosphorylation is poor [17,21]. Thus, it would appear that elimination of propylene from the diisopropoxy moieties and fragmentation of the peptide backbone provide energetically more acceptable routes for use of the collision energy than does dephosphorylation of the tyrosine.

The spectrum in Fig. 5 contains a number of major peaks that were not used in the analysis of the DFP–YTK adduct. Most of these are attributable to fragments from a second albumin peptide. The doubly charged form of LVNEVTEFAK appears at *m/z* 575. The peaks at 218, 365, 494, and 595 amu (plus additional peaks at the higher masses of 694, 823, 937, and 1036 amu) correspond to the complete *y*-series for this contaminating peptide. Peaks at 213, 327, 456, and 555 amu can be assigned to a *b*-series from this same peptide, whereas peaks at 312, 343, 426, and 444 amu reflect internal fragments. The large peak at 86 amu is the immonium ion of Leu/Ile.

The presence of two isopropyl groups in parent ion 575.4 supports the conclusion that the DFP–albumin adduct does not age.

The unique set of six phosphorylated fragment ions in Fig. 5 for DFP–albumin could be useful for identifying exposure to DFP in an MS method that selectively searches for characteristic fragment ions.

Only one covalent binding site for OP

Human albumin labeled with dichlorvos or DFP and digested with pepsin was searched for additional peptides that might bind OP. MALDI-TOF and Q-Trap analysis revealed no other OP adducts. The only identified OP binding site was Tyr411.

Discussion

Mechanism of OP labeling of albumin

Each of the OP toxicants tested in this work labeled Tyr411 of human albumin. The stoichiometry of labeling has been shown to be 1 mol ^{3}H[DFP] or ^{14}C[DFP] incorporated per mole of albumin [3–5,12]. The specific labeling of 1 tyrosine in a molecule that contains 18 tyrosines suggests that Tyr411 is in a special environment. This tyrosine has an unusually low pK_a of 7.9 to 8.3 [3,22], in contrast to the pKa of 10 for the average tyrosine.

Tyr411 is the active site residue not only for reaction with OP but also for reaction with esters such as *p*-nitrophenyl acetate, carbamates such as carbaryl, and amides

such as *o*-nitroacetanilide [3,23–25]. The esterase and amidase activity of albumin can be inhibited by pretreatment with DFP. Conversely, labeling with DFP can be prevented by pretreatment with *p*-nitrophenyl acetate, which forms a stable acylated albumin adduct [3]. The sensitivity of albumin esterase activity to ionic strength led Means and Wu to conclude that the reactive tyrosine residue is located on the surface of albumin in an apolar environment adjacent to several positively charged groups [3]. This description of the OP binding site of albumin was proven to be correct when the crystal structure was solved [26,27]. Subdomain IIIa of albumin contains a pocket lined by hydrophobic side chains. The hydroxyl of Tyr411 is close to the side chains of Arg410 and Lys414.

Site-directed mutagenesis experiments have shown that albumin esterase activity is abolished when Tyr411 is mutated to Ala and is severely diminished when Arg410 is mutated to Ala [28]. These results support Tyr411 as the active site for albumin esterase activity and support a role for Arg410 in stabilizing the reactive anionic form of Tyr411. The negatively charged Tyr411 is available for nucleophilic attack on ester and amide substrates. Although crystal structures of several ligand albumin complexes have been solved [29], the crystal structure of an OP–albumin adduct is not yet available.

No aging of OP–albumin adducts

Aging of OP-labeled acetylcholinesterase and butyrylcholinesterase is defined as the loss of an alkoxy group from the OP-labeled active site serine [30,31]. The nerve agents sarin, soman, and VX yield the same aged OP derivative, so that these agents may be difficult to distinguish when bound to acetylcholinesterase or butyrylcholinesterase [32,33].

Three of the OP agents studied in this work—sarin, DFP, and chlorpyrifos oxon—are known to age when bound to acetylcholinesterase and butyrylcholinesterase. However, these OP toxicants did not age when bound to albumin. Aging is a catalytic process that requires the participation of nearby histidine and glutamic acid residues [34]. Residues that promote aging are not present in the active site pocket of albumin. The absence of aging allows the bound OP to be spontaneously released from Tyr411. This makes albumin an OP hydrolase, albeit a very slow one. Albumin hydrolyzes chlorpyrifos oxon, *O*-hexyl *O*-2,5-dichlorophenylphosphoramidate, and paraoxon [10–13]. We recently measured the hydrolysis of soman by human albumin and found a deacylation rate of 0.0052 per hour (unpublished).

The observation that OP–albumin adducts do not age is supported by the findings of others [8,9]. Using liquid chromatography–mass spectrometry (LC–MS), Black and coworkers found *O*-(pinacolyl methylphosphonyl)tyrosine in human plasma as well as in albumin samples that had been treated with soman, and they found *O*-(isopropyl methylphosphonyl)tyrosine in samples treated with sarin [8]. If aging had occurred, the products would have been (methylphosphonyl)tyrosine for both soman and sarin. Adams and coworkers used gas chromatography–mass spectrometry (GC–MS) to measure sarin and soman recovered from human plasma and albumin samples [9]. The plasma and albumin were reacted with sarin or soman, excess agent was removed by solid phase extraction, and the samples were treated with potassium fluoride to release the bound OP. Intact sarin and soman were recovered, demonstrating that aging had not occurred. The absence of aging in OP–albumin adducts suggests that albumin could be a useful biomarker to distinguish between soman and sarin exposure. In the same manner, OP–albumin adducts could distinguish between pesticide and nerve agent exposure.

Advantages and disadvantages of MALDI-TOF–MS

The MALDI-TOF–mass spectrometer is an easy instrument to use. Samples need to be free of salt, and the concentration of peptide needs to be approximately 1 pmol/μl. As little as 0.5 μl of a 1-pmol/μl solution gives a good signal. Results are acquired in seconds.

The disadvantage of MALDI is that not all peptides ionize when the sample contains a mixture of peptides. For example, the FP-biotin-labeled bovine albumin YTR peptide gave an intense signal at 1012 amu (data not shown). In contrast, FP-biotin-labeled human albumin YTK peptide gave no signal. In both experiments, the sample was a mixture of tryptic peptides. Ion suppression is a common problem in MS, and one way of solving the problem is to separate the peptide of interest from other peptides by HPLC before examining it by MALDI–TOF MS. This strategy allowed us to detect sarin-labeled albumin in human plasma. Alternatively, the peptides can be separated by step elution from a C18 ZipTip, a procedure that was successful for nerve agent adducts of acetylcholinesterase [35].

OP–albumin as a biomarker of OP exposure

Many proteins in human plasma are labeled by OP. MS assays have been developed for OP–butyrylcholinesterase adducts [36–38]. The current article has provided an assay for OP–albumin adducts. Albumin is far less reactive with OP than butyrylcholinesterase, but the 10,000-fold higher concentration of albumin in plasma compared with butyrylcholinesterase (40,000–50,000 vs. 4–5 mg/ml) means that both albumin and butyrylcholinesterase will be labeled when a person is exposed to OP. New assays that use precursor and fragment ion *m*/*z* values in selected reaction monitoring experiments are expected to be capable of diagnosing low-dose exposure [38].

Antibody to OP–albumin

The information presented in this article could have application to the monitoring of individuals exposed to

OP. It may be possible to detect exposure through the use of an antibody detection assay directed toward the OP–albumin adduct at Tyr411. There is precedent for the generation of antibodies to very small haptens bound to protein. For example, antibodies that distinguish among phosphotyrosine, phosphoserine, and phosphothreonine have been successfully produced [39,40]. Antibodies to soman, sarin, and VX bound to carrier proteins through a chemical linker have been produced [41–43]. The proposed OP–albumin epitope could be more useful for detection of OP exposure than existing antibodies because the OP–albumin adduct has no chemical linker and no foreign protein environment.

An OP–albumin adduct at Tyr411 may generate an antibody response in exposed individuals, and the antibody could be detected to determine a history of exposure to OP. This would facilitate monitoring exposure to OP long after the exposure incident and long after the antigen has disappeared.

Acknowledgments

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Albumin, a New Biomarker of Organophosphorus Toxicant Exposure, Identified by Mass Spectrometry

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The classical laboratory tests for exposure to organophosphorus toxicants (OP) are inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity in blood. In a search for new biomarkers of OP exposure, we treated mice with a biotinylated organophosphorus agent, FP-biotin. The biotinylated proteins in muscle were purified by binding to avidin-Sepharose, separated by gel electrophoresis, digested with trypsin, and identified from their fragmentation patterns on a quadrupole time-of-flight mass spectrometer. Albumin and ES1 carboxylesterase (EC 3.1.1.1) were found to be major targets of FP-biotin. These FP-biotinylated proteins were also identified in mouse plasma by comparing band patterns on nondenaturing gels stained for albumin and carboxylesterase activity, with band patterns on blots hybridized with Streptavidin Alexa-680. Two additional FP-biotin targets, AChE (EC 3.1.1.7) and BChE (EC 3.1.1.8), were identified in mouse plasma by finding that enzyme activity was inhibited 50–80%. Mouse plasma contained eight additional FP-biotinylated bands whose identity has not yet been determined. *In vitro* experiments with human plasma showed that chlorpyrifos oxon, echothiophate, malaoxon, paraoxon, methyl paraoxon, diazoxon, diisopropylfluorophosphate, and dichlorvos competed with FP-biotin for binding to human albumin. Though experiments with purified albumin have previously shown that albumin covalently binds OP, this is the first report of OP binding to albumin in a living animal. Carboxylesterase is not a biomarker in man because humans have no carboxylesterase in blood. It is concluded that OP bound to albumin could serve as a new biomarker of OP exposure in man.

Key Words: **acetylcholinesterase; butyrylcholinesterase; organophosphate; FP-biotin; albumin.**

Organophosphorus toxicants (OP) are used in agriculture as pesticides, in medical practice as antihelminthics, in the airline industry as additives to hydraulic fluid and jet engine oil, and as chemical warfare agents. These compounds are known to exert their acute effects by inhibiting acetylcholinesterase (EC 3.1.1.7, AChE). The excess acetylcholine that accumulates causes an imbalance in the nervous system that can result in death (McDonough and Shih, 1997).

Though AChE is the clinically important target of OP exposure, other proteins also form a covalent bond with OP, depending on the identity of the OP (Casida and Quistad, 2004). These secondary targets include butyrylcholinesterase, acylpeptide hydrolase, neurotoxic esterase, fatty acid amide hydrolase, arylformamidase, cannabinoid CB1 receptor, muscarinic acetylcholine receptor, and carboxylesterase. With the exception of neurotoxic esterase, whose inhibition is responsible for delayed neuropathy, the toxicological relevance of inhibition of these secondary targets is not yet understood (Casida and Quistad, 2004; Ray and Richards, 2001). Albumin has not previously been shown to bind OP in a living animal, though *in vitro* experiments with purified albumin have demonstrated covalent binding to diisopropylfluorophosphate, sarin, and soman (Black *et al*., 1999; Means and Wu, 1979; Murachi, 1963; Sanger, 1963; Schwartz, 1982). Albumin hydrolyzes chlorpyrifos oxon and paraoxon (Ortigoza-Ferado *et al*., 1984; Sultatos *et al*., 1984).

The toxic effects of a particular OP cannot be attributed entirely to inhibition of AChE. Toxic signs are different for each OP when that OP is administered at low doses (Moser, 1995). For example, a low dose of fenthion decreased motor activity in rats by 86% but did not alter the tail-pinch response, whereas a low dose of parathion did not affect motor activity but did decrease the tail-pinch response (Moser, 1995). These toxicological observations suggest that OP have other biological actions in addition to their cholinesterase-inhibitory properties.

Another confounding observation is the finding that toxic signs do not correlate with degree of AChE inhibition. Rats given doses of OP that inhibited AChE to similar levels had more severe toxicity from parathion than chlorpyrifos (Pope, 1999). There are also examples of toxic signs unaccompanied by AChE inhibition. Workers who manufacture the OP pesticide quinalphos have significantly low scores for memory, learning ability, vigilance, and motor response, though their blood AChE activity levels are the same as in control subjects (Srivastava *et al*., 2000). Chronic low-level exposure to OP

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induces neuropsychiatric disorders without inhibition of esterase activity (Ray and Richards, 2001; Salvi *et al*., 2003). These observations have led to the suggestion that some OP have toxicologically relevant sites of action in addition to AChE (Moser, 1995; Pope, 1999; Ray and Richards, 2001). The hypothesis arose that a given OP reacts not only with AChE, but with a set of proteins unique for each OP.

Our goal is to identify the proteins in a living animal that covalently bind the biotin-tagged OP called FP-biotin (Kidd *et al*., 2001; Liu *et al*., 1999). In this report we used tandem mass spectrometry, enzyme activity assays, gel electrophoresis, and blots to identify four FP-biotin-labeled proteins in the muscle and plasma of mice that had been injected with FP-biotin ip. We found FP-biotin-labeled albumin, carboxylesterase, BChE, and AChE. This is the first report to demonstrate that albumin is a significant target of OP binding in a living animal. Eight other proteins in mouse blood became labeled but have not yet been identified.

MATERIALS AND METHODS

Materials. FP-biotin (MW 592.3) was custom synthesized by Troy Voelker in the laboratory of Charles M. Thompson at the University of Montana (Liu *et al*., 1999). Purity was checked by NMR and mass spectrometry, and no evidence of contamination was detected. FP-biotin was stored as a dry powder at − 70°C. Just before use, the dry powder was dissolved in 100% ethanol to a concentration of 13.3 mg/ml and diluted with saline to 15% ethanol containing 2 mg/ml FP-biotin.

Immun-Blot PVDF membrane for protein blotting, 0.2 μm (catalog #162-0177) and biotinylated molecular weight markers (catalog #161–0319) were from Bio-Rad Laboratories, Hercules, CA. Streptavidin Alexa-680 fluorophore (catalog #S-21378) was from Molecular Probes, Eugene, OR. Avidin-agarose beads (catalog # A-9207), iso-OMPA, and bovine albumin, essentially fatty acid-free (Sigma A 7511) were from Sigma-Aldrich, St. Louis, MO. Echothiophate iodide was from Wyeth-Ayerst, Rouses Point, NY. All other OP were from Chem Service Inc, West Chester, PA.

Mice. The Institutional Animal Care and Use Committee of the University of Nebraska Medical Center approved all procedures involving mice. Animal care was provided in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice completely lacking AChE protein were made by gene targeting (Xie *et al*., 2000) at the University of Nebraska Medical Center. Exons 2, 3, 4, and 5 of the ACHE gene were deleted to make AChE–/– mice. The AChE–/– animals are in strain 129Sv genetic background. The colony is maintained by breeding heterozygotes because AChE–/– mice do not breed (Duysen *et al*., 2002). Wild-type mice are littermates of AChE–/– mice. The strain 129Sv mice were used for experiments with FP-biotin.

Injection of FP-biotin into mice. Mice were injected intraperitoneally with FP-biotin dissolved in 15% ethanol to give a dose of 56 or 5 mg/kg, or with vehicle alone. The dose of FP-biotin was calculated from dry weight. No correction was made for the fact that FP-biotin is a mixture of phosphorus stereoisomers. Mice were euthanized 120 min after FP-biotin injection, by inhalation of carbon dioxide. Blood was washed out of tissues by intracardial perfusion with saline solution. Tissues from six mice were analyzed by mass spectrometry: two AChE–/– FP-biotin treated (5 and 56 mg/kg), two AChE–/– untreated, one wild-type FP-biotin treated (56 mg/kg), and one wild-type untreated. In addition wild-type mice were treated with 0, 0.5, 1.0, 5.0, or 18.8 mg/kg FP-biotin ($n = 2$ for each dose). Blood was analyzed by gel electrophoresis, blotting, and enzyme activity assays.

Enzyme activity. AChE activity was measured with 1 mM acetylthiocholine in the presence of 0.5 mM dithiobisnitrobenzoic acid (Ellman *et al*., 1961) after inhibiting BChE activity for 30 min with 0.1 mM iso-OMPA, in 0.1 M potassium phosphate pH 7.0, at 25°C. BChE activity was measured with 1 mM butyrylthiocholine. $\Delta E_{412nm} = 13{,}600\ M^{-1}\ cm^{-1}$ at pH 7.0. Carboxylesterase activity was measured with 5 mM *p*-nitrophenyl acetate after inhibiting AChE and BChE with 0.01 mM eserine, and after inhibiting paraoxonase with 12.5 mM EDTA. $\Delta E_{400nm} = 9000\ M^{-1}\ cm^{-1}$ at pH 7.0. AChE, BChE, and carboxylesterase units of activity are micromoles hydrolyzed per minute at pH 7.0, 25°C.

Isolation of FP-biotin-labeled protein. To prepare OP-labeled proteins for mass spectrometry, FP-biotin-labeled proteins in muscle were purified on avidin-agarose beads and separated by SDS–polyacrylamide gel electrophoresis. Proteins from mice that had received no FP-biotin were purified by the same procedure.

Tissues were homogenized in 10 volumes of 50 mM TrisCl pH 8.0 containing 5 mM EDTA, and centrifuged for 10 min in a microfuge at 12,000 rpm to partially clarify the suspension. A detailed example of the protocol follows. The 0.96 ml of muscle homogenate (7.4 mg protein/ml) was diluted with 3.75 ml of 50 mM TrisCl pH 8.0, 5 mM EDTA to make 1.5 mg/ml protein solution. SDS was added to make the solution 0.5% SDS. The protein solution was heated for 3 min in a boiling water bath and then diluted with buffer to make the final SDS concentration 0.2%. The protein solution was incubated with 100 μl of washed avidin-agarose beads (1.9 mg avidin/ml of beads) overnight at room temperature, with continuous inversion, to bind the FP-biotin-labeled proteins to the beads. Beads were washed three times with the TrisCl/EDTA buffer, containing 0.2% SDS, to remove nonspecifically bound protein. Twenty-five μl of 6× SDS–PAGE loading buffer (0.2 M TrisCl, pH 6.8, 10% SDS, 30% glycerol, 0.6 M dithiothreitol and 0.012% bromophenol blue) were added to the 100 μl of beads, and the mixture was heated at 85°C for 3 min. This step released the biotinylated proteins from the avidin beads. Equal amounts of the bead mixture were loaded directly into two wells of a 10–20% gradient SDS–PAGE (10-well format, 1.5-mm thick) and run for 4000 volt-hours in the cold room. The gel was stained with Coomassie blue G250 (Bio-Safe from BioRad), and destained with water. Coomassie G250 is reportedly 2–8 fold more sensitive than Coomassie R250 (BioRad specifications). To minimize contamination from keratin, the staining dish had been cleaned with sulfuric acid, and the water was Milli-Q purified. For the same reason, gloves were worn for all operations involving the gel.

Protein digestion protocol. The proteins separated on SDS–PAGE were digested with trypsin to prepare them for identification by mass spectral analysis. Gloves were worn throughout these procedures, all solutions were made with Milli-Q purified water, and all glassware, plasticware, and tools were rinsed with Milli-Q purified water to minimize keratin contamination. Each Coomassie-stained band from one lane of the SDS–PAGE was excised, placed into a separate 1.5-ml microfuge tube, and chopped into bits. The amount of gel excised was kept to a minimum. The gel bits were destained by washing with 200 μl of 25 mM ammonium bicarbonate (Aldrich) in 50% acetonitrile (synthesis grade, from Fisher). After three washes, the gel bits were colorless and had shrunken considerably. Residual liquid was removed, and the gel bits dried by evaporation in a Speedvac (Jouan). Disulfide bonds in the protein were reduced by incubating the gel bits with 10 mM dithiothreitol (molecular biology grade, from Sigma) in 200 μl of 100 mM ammonium bicarbonate for 1 h at 56°C. The gel pieces were then centrifuged, excess solution was removed, and the protein was alkylated with 55 mM iodoacetamide (Sigma) in 120 μl of 100 mM ammonium bicarbonate for 1 h at room temperature in the dark. The gel bits were again centrifuged, excess solution was removed, and the bits were washed with 200 μl of 25 mM ammonium bicarbonate in 50% acetonitrile (three times). Residual liquid was again removed and the gel bits dried by evaporation in the Speedvac. The proteins were digested in the gel with trypsin, using 12.5 ng/μl of sequencing grade trypsin (Promega) in 25 mM ammonium bicarbonate. Ninety μl of the trypsin solution were added to the dry gel bits and incubated at 4°C for 20 min, to allow the gel to re-swell. Then 60 μl of 25 mM ammonium bicarbonate were layered over each sample, and the samples were incubated at 37°C overnight (about 17 h). Peptides were extracted by incubating each reaction mixture with 200 μl of 0.1% trifluoroacetic acid (sequencing grade from Beckman) in 60% acetonitrile for one h at

room temperature. Extraction was repeated three times, and the extracts for each sample were pooled. The pooled extracts were evaporated to dryness in the Speedvac, and the dry samples were stored at −20°C until analyzed.

Mass spectral analysis. Each tryptic peptide digest was resuspended in 40 µl of 5% aqueous acetonitrile/0.05% trifluoroacetic acid. A 10-µl aliquot of the digest was injected into a CapLC (capillary liquid chromatography system from Waters Corp) using 5% aqueous acetonitrile/0.05% trifluoroacetic acid (auxiliary solvent) at a flow rate of 20 µl per min. Peptides were concentrated on a C_{18} PepMap™ Nano-Precolumn™ (5 mm × 0.3 mm id, 5µm particle size) for 3 min, and then eluted onto a C_{18} PepMap™ capillary column (15 cm × 75 µm id, 3 µm particle size both from LC Packings), using a flow rate of 200–300 nl per min. Peptides were partially resolved using gradient elution. The solvents were 2% aqueous acetonitrile/0.1% formic acid (solvent A), and 90% acetonitrile/10% isopropanol/0.2% formic acid (solvent B). The solvent gradient increased from 5% B to 50% B over 22 min, then to 80% B over 1 min, and remained at 80% B for 4 min. The column was then flushed with 95% B for 3 min and equilibrated at 5% B for 3 min before the next sample injection.

Peptides were delivered to the Z-spray source (nano-sprayer) of a Micromass Q-TOF (tandem quadrupole/time-of-flight mass spectrometer from Waters Corp.) through a 75-µm id capillary, which connected to the CapLC column. In order to ionize the peptides, 3300 volts were applied to the capillary, 30 volts to the sample cone, and zero volts to the extraction cone. Mass spectra for the ionized peptides were acquired throughout the chromatographic run, and collision-induced dissociation spectra were acquired on the most abundant peptide ions (having a charge state of 2+, 3+, or 4+). The collision-induced dissociation spectrum is unique for each peptide and is based on the amino acid sequence of that peptide. For this reason, identification of proteins using collision-induced dissociation data is superior to identification by only the peptide mass fingerprint of the protein. The collision cell was pressurized with 1.5 psi ultrapure argon (99.999%), and collision voltages were dependent on the mass-to-charge ratio and the charge state of the parent ion. The time-of-flight measurements were calibrated daily using fragment ions from collision-induced dissociation of [Glu^1]-fibrinopeptide B. Each sample was post-processed using this calibration and Mass Measure (Micromass). The calibration was adjusted to the exact mass of the autolytic tryptic fragment at 421.76, found in each sample.

The mass and sequence information for each detected peptide was submitted either to ProteinLynx Global Server 1.1 (a proprietary software package, from Micromass), or to MASCOT (a public access package provided by Matrix Science at http://www.matrix-science.com). Data were compared to all mammalian entries (ProteinLynx) or just mouse entries (MASCOT) in the NCBInr database (National Center for Biotechnology Information). Search criteria for ProteinLynx were set to a mass accuracy of 0.25 Da, fixed modification of methionine (oxidation), and variable modification of cysteine (carbamidomethylation). One missed cleavage by trypsin was allowed. Search criteria for MASCOT were set to a mass accuracy of ±0.1 Da, one missed cleavage, variable modification of methionine (oxidation) and cysteine (carbamidomethylation), and peptide charge +2 and +3. Both software packages calculated a score for each identified protein based on the match between the experimental peptide mass and the theoretical peptide mass, as well as between the experimental collision-induced dissociation spectra and the theoretical fragment ions from each peptide. Results were essentially the same from both packages.

Polyacrylamide gel electrophoresis. Gradient polyacrylamide gels (4–30%) were cast in a Hoefer gel apparatus. Electrophoresis was for 5000 volt-hours (200 volts for 25 h) at 4°C for nondenaturing gels and 2500 volt-hours (100 volts for 25 h) at 4°C for gels containing 0.1% SDS.

Staining gels for BChE activity. Nondenaturing gels were stained for BChE activity by the method of Karnovsky and Roots (1964). The staining solution contained 180 ml of 0.2 M sodium maleate pH 6.0, 15 ml of 0.1 M sodium citrate, 30 ml of 0.03 M cupric sulfate, 30 ml of 5 mM potassium ferricyanide, and 0.18 g butyrylthiocholine iodide in a total volume of 300 ml. Gels were incubated, with shaking, at room temperature for 3 to 5 h. The reaction was stopped by washing the gels with water. To determine the location of albumin, activity-stained gels were stained with Coomassie blue.

Staining gels for carboxylesterase activity and albumin. Nondenaturing gels were incubated in 100 ml of 50 mM TrisCl pH 7.4 in the presence of 50 mg beta-naphthylacetate dissolved in 1 ml ethanol, and 50 mg of solid Fast Blue RR. The naphthylacetate precipitates when it is added to the buffer, but enough remains in solution that the reaction works. Though the Fast Blue RR does not dissolve, pink to purple bands develop on the gel within minutes (Nachlas and Seligman, 1949). A maximum of 30 min incubation at room temperature was needed. The gels were washed with water and photographed. This stain is primarily for carboxylesterase. Albumin gives a faint band with this method because albumin slowly hydrolyzes beta-naphthylacetate (Tove, 1962). Activity-stained gels were counterstained with Coomassie blue to verify the location of albumin.

To align bands on gels stained for enzyme activity with biotinylated bands on a PVDF membrane, the transparent activity-stained gels were placed on top of a printed image of the fluorescent bands in the PVDF membrane.

Visualizing FP-biotin-labeled proteins. For determination of the number and size of proteins labeled by FP-biotin, proteins were subjected to gel electrophoresis, transfer to a PVDF membrane, and hybridization with a fluorescent probe. The details of the procedure follow.

Proteins were transferred from the polyacrylamide gel to PVDF membrane (Immun-Blot from BioRad) electrophoretically in a tank using plate electrodes (TransBlot from BioRad), at 0.5 amps, for 1 h, in 3 l of 25 mM Tris/192 mM glycine buffer, pH 8.2, in the cold room (4°C), with stirring. The membrane was blocked with 3% gelatin (BioRad) in 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl for 1 h at room temperature. The 3% gelatin solution had been prepared by heating the gelatin in buffer in a microwave oven for several seconds. The blocked membrane was washed three times with 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl and 0.05% Tween-20, for 5 min.

Biotinylated proteins were labeled with 9.5 nM Streptavidin Alexa-680 fluorophore in 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl, 0.05% Tween-20, 0.2% SDS, and 1% gelatin, for 2 h, at room temperature, protected from light. Shorter reaction times resulted in less labeling. The SDS was found to increase the intensity of labeling. The membrane was washed twice with 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl and 0.05% Tween-20, and twice with 20 mM TrisCl buffer, pH 7.5, containing 0.5 M NaCl, for 20 min each, while protected from light.

Membranes were scanned with the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) at 42 microns per pixel. The Odyssey employs an infrared laser to excite a fluorescent probe, which is attached to the target protein, and then collects the emitted light. The emitted light intensity is directly proportional to the amount of probe. Both the laser and the detector are mounted on a moving carriage positioned directly below the membrane. The membrane can be scanned in step sizes as small as 21 microns, providing resolution comparable to X-ray film. Data are collected using a 16-bit dynamic range. The fluorophore is stable in the laser, making it possible to scan the membrane repeatedly, while using different intensity settings to optimize data collection for both strong and weak signals. The membrane was kept wet during scanning.

Biotinylated protein standards. The biotinylated bovine serum albumin (BSA) standard was prepared by incubating 10 µM BSA (0.5 mg/ml) with 20 µM FP-biotin in 20 mM TrisCl pH 7.4 at room temperature for 16 h. The biotinylated BChE standard was prepared by incubating 50 nM human BChE (3 units/ml; 4.2 µg/ml) with 10 µM FP-biotin, in 20 mM TrisCl pH 7.4 at room temperature for 16 h.

Amount of biotinylated albumin in mouse plasma. The percentage of FP-biotinylated albumin in mouse plasma was estimated from the relative intensities of the biotinylated albumin band and the biotinylated BChE band on a blot stained with Streptavidin Alexa-680. The concentration of BChE in mouse plasma is 0.003 mg/ml. The concentration of biotinylated BChE was calculated from the reduction in enzyme activity. The concentration of albumin in mouse plasma is 50 mg/ml. These values allowed estimation of percent biotinylated albumin in plasma. For example, when BChE activity was inhibited 35%, the biotinylated BChE band represented about 0.001 mg/ml biotinylated BChE. A biotinylated albumin band of similar intensity would contain 0.001 mg/ml biotinylated albumin. When mouse plasma had to be diluted

1000-fold to reduce the intensity of biotinylated albumin to a similar intensity as the biotinylated BChE in undiluted plasma, it was calculated that the concentration of biotinylated albumin in undiluted plasma was 1 mg/ml.

Binding various OP to human albumin. Human plasma was diluted 1:100 to reduce the albumin concentration to 10 μM. The diluted plasma was reacted *in vitro* with 10 mM malaoxon, paraoxon, chlorpyrifos oxon, methyl paraoxon, dichlorvos, diisopropylfluorophosphate diazoxon, echothiophate, or iso-OMPA for 1 h in 20 mM TrisCl pH 7.5 at 25°C. Then FP-biotin was added to 10 μM and allowed to react for 1 h, and 10 μl containing the equivalent of 0.1 μl plasma was loaded per lane on a nondenaturing gel. Biotinylated proteins were visualized with Streptavidin Alexa-680 after transfer to PVDF membrane.

Statistical analysis. Samples were analyzed by independent samples *t*-test assuming equal variances. Probability values less than 0.05 were considered significant.

RESULTS

Toxicity of FP-Biotin

The structure of FP-biotin is given in Figure 1. A dose of 56 mg/kg FP-biotin ip was lethal to AChE–/– mice and reduced plasma BChE activity from 1.9 ± 0.4 units/ml in untreated animals to 0.006 ± 0.003 units/ml post treatment, a 99.7% inhibition. A dose of 18.8 mg/kg ip was not lethal, but did cause severe cholinergic signs of toxicity. This dose reduced plasma BChE activity to 0.34 ± 0.09 units/ml, an 82% inhibition. A dose of 5 mg/kg caused only mild signs of toxicity and inhibited plasma BChE of AChE–/– mice 37%, to 1.2 ± 0.4 units/ml. In contrast, wild-type mice showed no signs of toxicity after treatment with 18.8 or 5 mg/kg FP-biotin ip, even though their plasma BChE activity was inhibited to the same extent as in the AChE–/– mice.

Plasma AChE activity in wild-type mice treated with 18.8 mg/kg FP-biotin was inhibited from a predose level of 0.30 ± 0.01 units/ml to 0.13 units/ml, a 56% inhibition. There was no inhibition at lower doses. AChE activity was not measured in AChE–/– mice, because these knockout animals have no AChE activity (Xie *et al*., 2000). AChE has a 10-fold lower affinity for FP-biotin compared to BChE (Schopfer, unpublished). This explains why a given dose of FP-biotin caused less inhibition of AChE than of BChE.

Plasma carboxylesterase activity was inhibited to the same extent in AChE–/– and +/+ mice. A dose of 18.8 mg/kg FP-biotin caused a reduction from the untreated values of 18.6 ± 0.6 units/ml to 3.5 units/ml, an 81% inhibition, while a dose of 5 mg/kg reduced the carboxylesterase levels to 9.2 ± 1.6 units/ml, a 50% reduction.

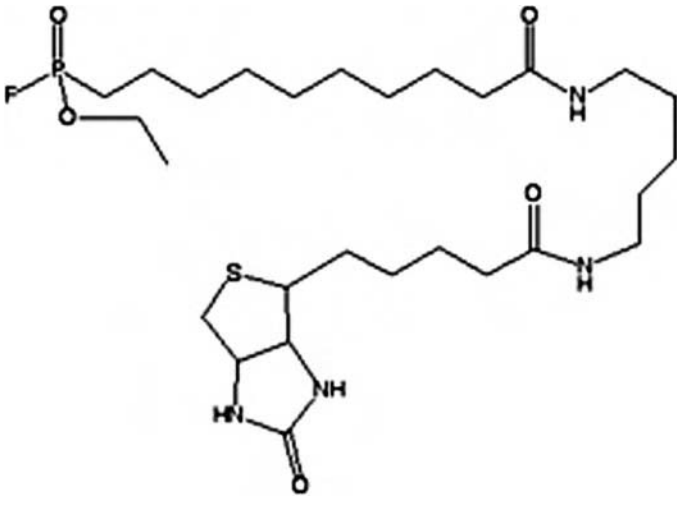

FIG. 1. Structure of FP-biotin. The OP has a reactive phosphonofluoridate group tethered to biotin via a spacer arm.

Fp-Biotin Does Not Cross the Blood–Brain Barrier

FP-biotin treated AChE–/– mice showed no inhibition of BChE in brain, supporting the conclusion that FP-biotin does not cross the blood–brain barrier.

Identification of FP-Biotinylated Proteins by Mass Spectrometry

Muscle proteins from mice that had been treated with FP-biotin, as well as from untreated control mice, were isolated by binding to avidin beads. The proteins were released from avidin by boiling in SDS and separated by SDS gel electrophoresis. Protein bands visible with Coomassie blue staining were excised and digested with trypsin. Fragmentation of tryptic peptides yielded amino acid sequence information characteristic of the protein. The proteins listed in Table 1 were consistently identified in three separate experiments. The probability score for correct identification (MOWSE score) was extremely high at 1157 for albumin and 655 for ES1 carboxylesterase. A MOWSE score of 69 is significant ($p < 0.05$), so scores of 1157 and 655 show complete confidence. Though albumin and ES1 carboxylesterase were found in samples prepared from muscle, these proteins are typically expressed at high levels in

TABLE 1

Mass Spectral Identification of Proteins in Muscle That Became Biotinylated After Treatment of Mice with FP-Biotin

Protein	MW, kDa	Genbank #	MOWSE score	% Cover	# Peptides identified
Albumin	67	Gi5915682	1157	51	17
Es1 carboxylesterase	61	Gi22135640	655	40	11

Note. MW is the protein molecular weight in kilodaltons. Genbank # is the protein accession number in the computerized databank accessible through PubMed. MOWSE score: Molecular Weight Search, a measure of the probability of a match between the experimental data and the peptide mass in the database. MOWSE scores greater than 69 are significant ($p < 0.05$). % Cover is the percent of the protein represented by the sequenced peptides. # Peptides identified is the number of peptides whose sequence matched albumin or ES1 carboxylesterase. The enzyme commission number for carboxylesterase is EC 3.1.1.1.

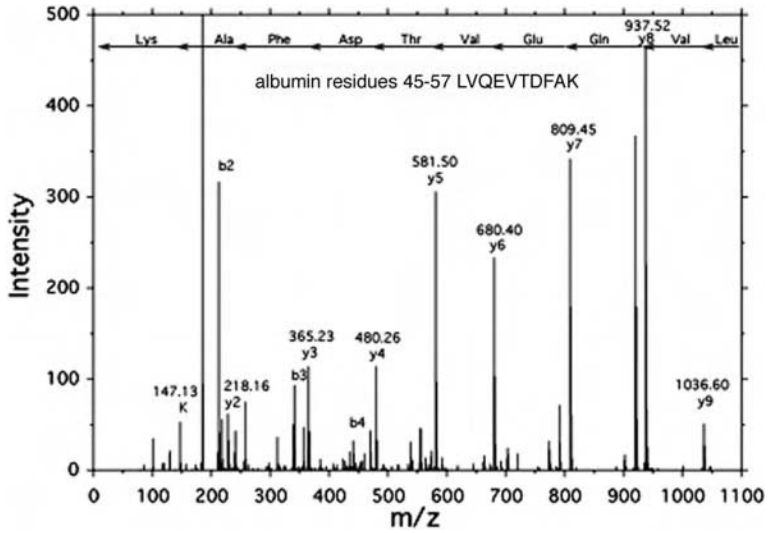

FIG. 2. Identification of albumin by mass spectrometry. The collision-induced dissociation mass spectrum of peptide Leu Val Gln Glu Val Thr Asp Phe Ala Lys of mouse albumin is shown. The parent ion has a mass-to-charge ratio (m/z) of 1149.64. The FP-biotinylated albumin was recovered from three mice treated with FP-biotin.

TABLE 2
Mass Spectral Identification of Biotinylated Proteins Extracted from Muscle of Untreated As Well As FP-Biotin Treated Mice

Protein	MW, kDa	Genbank #	EC #	MOWSE score	% Cover	# Peptides identified
Propionyl CoA carboxylase alpha	80	Gi29612536	6.4.1.3	1309	53	20
Pyruvate carboxylase	130	Gi464506	6.4.1.1	92	7	2
Methylcrotonyl CoA carboxylase alpha	79	Gi31980706	6.4.1.4	830	46	15

Note. EC# is the number assigned by the enzyme commission to identify the enzyme. Other abbreviations are defined in Table 1.

blood (Kadner *et al*., 1992; Peters, 1996). It is likely that they were transported from the blood into the extravascular fluid where they were not washed out by perfusion. Albumin was identified by 17 peptides. A representative mass spectrum of an albumin peptide is shown in Figure 2. These 17 peptides represented 51% of the mouse albumin sequence, leaving no doubt that albumin was labeled by FP-biotin. The untreated control tissues did not show albumin, thus demonstrating that only biotinylated albumin had bound to avidin beads. This control experiment eliminated the possibility that the albumin might have bound nonspecifically to the avidin beads or that albumin was endogenously biotinylated.

FP-biotinylated AChE and BChE were not found because these proteins are not abundant enough to give a Coomassie blue stained band on SDS gels. In this work only proteins that gave a Coomassie stained band were analyzed by mass spectrometry.

Identification of Endogenous Biotinylated Proteins

Endogenous biotinylated proteins were identified by mass spectrometry. The proteins in Table 2 were found in muscle of untreated as well as in FP-biotin treated mice. They are propionyl CoA carboxylase alpha, pyruvate carboxylase, and methylcrotonyl CoA carboxylase alpha. These endogenous biotinylated proteins bound to avidin beads and were abundant enough to be visualized as Coomassie blue bands on an SDS gel.

Blot Showing FP-Biotinylated Proteins in Mouse Plasma

A blot showing the proteins in plasma that became labeled with FP-biotin after treatment of mice with FP-biotin, is shown in Figure 3. Doses of 1 and 5 mg/kg FP-biotin were not toxic to the animals. The intense broad band in the middle of the gel resolved into three bands upon serial dilution of mouse plasma. The top band in the triplet is carboxylesterase (CE), the middle band is albumin, and the bottom band has not been identified.

A band for biotinylated BChE was visible in plasma from mice treated with 5 mg/kg but not 1 mg/kg FP-biotin. This is consistent with our finding that BChE activity was inhibited about 35% after treatment with 5 mg/kg but was not inhibited after treatment with 1 mg/kg FP-biotin.

In addition to albumin, carboxylesterase, and butyrylcholinesterase, mouse plasma contains about eight other biotinylated bands whose protein identity is unknown. The intensity of the

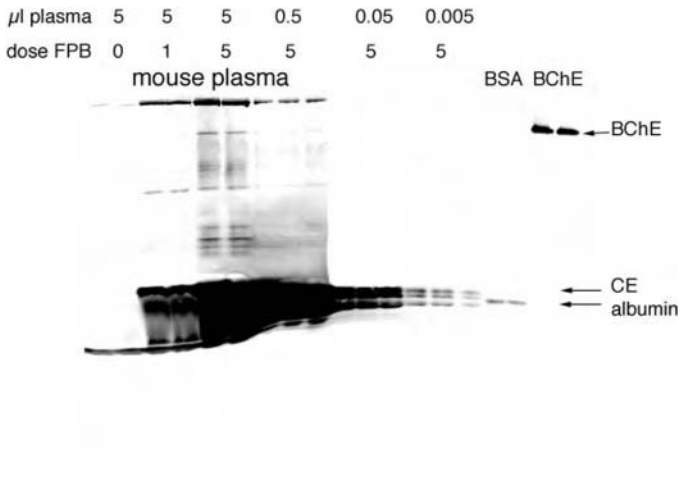

FIG. 3. FP-biotinylated proteins in mouse plasma visualized with Streptavidin Alexa-680. Mice were treated with 0, 1, or 5 mg/kg FP-biotin ip. Two h later blood was collected. Plasma proteins were separated on a nondenaturing 4–30% gradient polyacrylamide gel. Proteins were transferred to PVDF membrane, and biotinylated proteins were visualized by hybridizing the blot with Streptavidin Alexa-680. The volume of mouse plasma loaded per lane ranged from 5 to 0.005 μl. Two lanes marked BSA received 0.25 μg biotinylated BSA per lane. Two lanes marked BChE received 0.020 μg biotinylated BChE per lane.

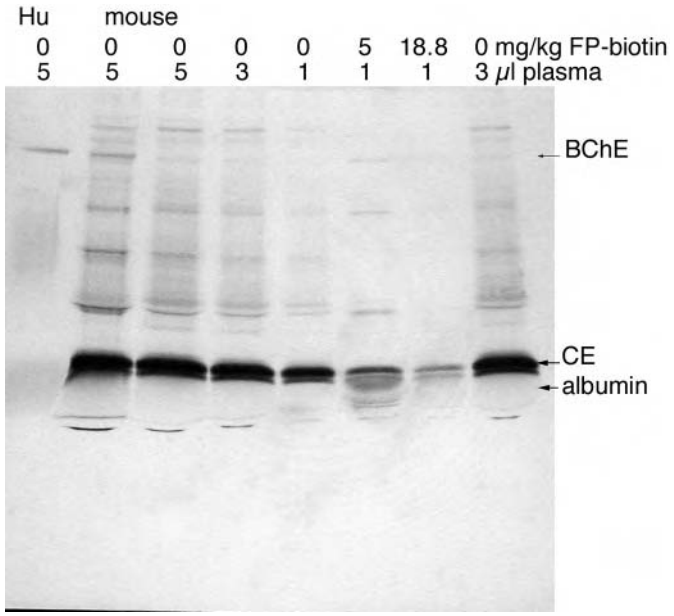

FIG. 4. Nondenaturing gel stained for carboxylesterase activity. Mice were treated with 0, 5, or 18.8 mg/kg FP-biotin ip. Blood was collected 2 h later; 1–5 μl of plasma was loaded per lane. The nondenaturing gel was stained for carboxylesterase activity. The intense band is carboxylesterase (CE). Albumin migrates immediately below carboxylesterase. The lane marked Hu received 5 μl of untreated human plasma. Note that human plasma contains no carboxylesterase.

band at the top of the gel is higher than that of mouse BChE, suggesting that this protein is more abundant than BChE and that it is highly reactive. The intensity of the other bands is equal to that of BChE or lower. Thus, mouse plasma contains at least 11 proteins that bind OP at physiological conditions, at doses of OP that produce no toxic signs.

About 1–2% of the albumin in mouse plasma is estimated to have bound FP-biotin in a mouse treated with 5 mg/kg FP-biotin ip. However, there is so much more albumin (50 mg/ml) than BChE (0.003 mg/ml) in mouse plasma that albumin consumes a significant amount of FP-biotin. Similar biotinylated band intensities were obtained in Figure 3 for BChE in undiluted plasma and for albumin diluted 1000-fold, suggesting that albumin bound 1000-fold more FP-biotin than was bound by BChE.

Activity-Stained Gels

The gels for Figures 3–5 were nondenaturing polyacrylamide gels. Nondenaturing gels were used because under these conditions the BChE tetramer of 340 kDa separated well from the 67 kDa albumin. This separation is not possible on SDS gels because albumin (67,000 MW) is 10,000 times more abundant in plasma than BChE (85,000 MW), and spreads into a broad band that overlaps with the BChE band, making it impossible to visualize BChE. A second reason for using nondenaturing gels is that nondenaturing gels allow identification of BChE and carboxylesterase based on activity. Figure 4 shows activity

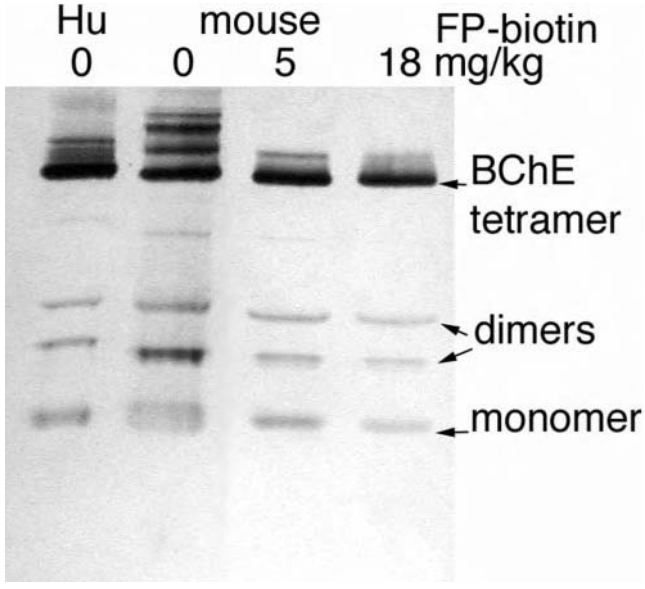

FIG. 5. Nondenaturing gel stained for BChE activity. Mice were treated with 0, 5, or 18.8 mg/kg FP-biotin ip. Blood was collected 2 h later. 5 μl of mouse plasma was loaded per lane. The lane marked Hu received 5 μl of untreated human plasma. Despite 35 to 80% inhibition of mouse BChE, enough BChE activity remained to give the characteristic pattern of BChE bands. The tetramer band contains about 95% of the total activity. Monomer and dimer bands are minor components. Note that the human and mouse BChE tetramer bands migrate to similar positions.

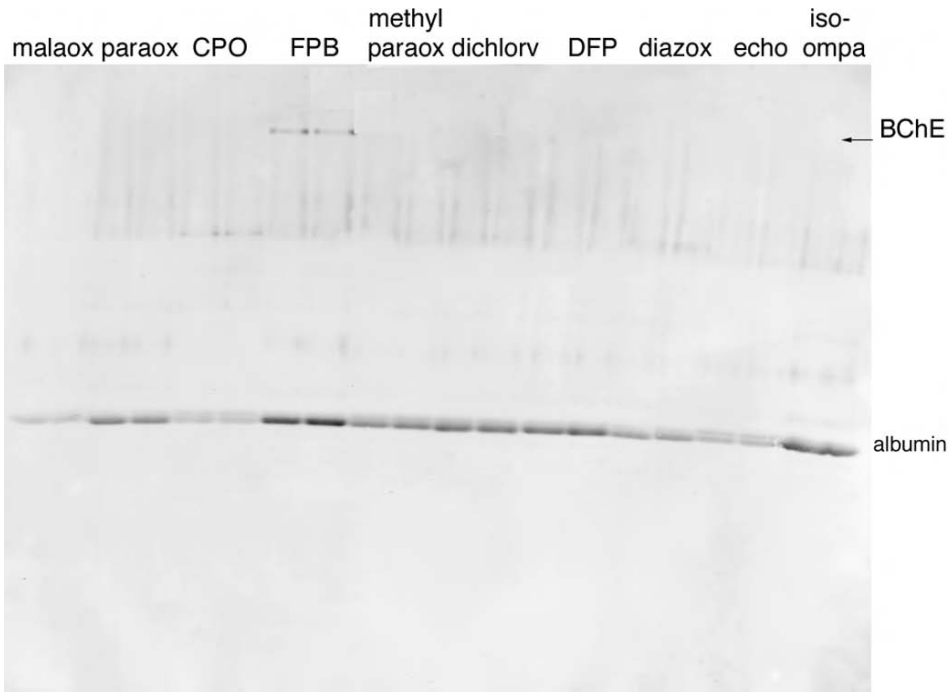

FIG. 6. Binding of FP-biotin to human albumin is inhibited by chlorpyrifos oxon and other OP. This experiment tests the ability of OP to block binding of FP-biotin to albumin. Human plasma, diluted 1:100, was incubated *in vitro* with malaoxon, paraoxon, chlorpyrifos oxon (CPO), methyl paraoxon, dichlorvos, diisopropylfluorophosphate (DFP), diazoxon, echothiophate (echo), or iso-OMPA for 1 h, before addition of FP-biotin (FPB). The lane marked FPB was treated only with FP-biotin. 10 µl containing the equivalent of 0.1 µl plasma was loaded per lane, in duplicate, on a nondenaturing gel. Biotinylated proteins were visualized with Streptavidin Alexa-680 after transfer to PVDF membrane. The intensity of the biotinylated albumin band was reduced by all OP except iso-OMPA indicating that all OP except iso-OMPA competed with FP-biotin for binding to human albumin.

with beta-naphthylacetate. The intense band is carboxylesterase (EC 3.1.1.1, CE) in mouse plasma. The bubble below carboxylesterase is albumin. BChE as well as several unidentified proteins also react with beta-naphthylacetate. Figure 5 shows activity of blood proteins with butyrylthiocholine. The BChE tetramer is the intense band near the top of the gel. By aligning bands in Figures 3, 4, and 5 we confirmed the identities of biotinylated albumin, carboxylesterase, and BChE in Figure 3.

Other OP Compete with FP-Biotin for Binding to Albumin

The goal of this experiment was to determine whether other OP bind to albumin. We wanted to know whether binding to albumin was a special property of FP-biotin or whether other OP also bound to albumin. If the OP binding site is a specific tyrosine (Black *et al*., 1999; Sanger, 1963) then pretreatment with other OP was expected to block binding of FP-biotin. Human plasma was used in this experiment because it does not contain carboxylesterase. The absence of carboxylesterase facilitated interpretation of the results, as there was no confusion between carboxylesterase and albumin. Figure 6 shows that other OP competed with FP-biotin for binding to albumin to varying extents. Pretreatment with chlorpyrifos oxon, echothiophate, malaoxon, paraoxon, methyl paraoxon, diazoxon, dichlorvos, and diisopropylfluorophosphate (DFP) reduced the binding of FP-biotin to human albumin *in vitro.* The only OP tested that gave no evidence of binding to albumin was tetraisopropylpyrophosphoramide (iso-OMPA).

These results suggest that OP binding to albumin is a general property of OP. It is to be noted that in this report FP-biotin has been demonstrated to bind to albumin from three species: mouse, bovine, and human. The albumins were in mouse plasma, purified bovine serum albumin, and human plasma.

DISCUSSION

OP Labels Proteins That Have No Active Site Serine

Organophosphorus toxicants are well known as inhibitors of serine proteases and serine esterases. Enzymes such as trypsin, chymotrypsin, thrombin, AChE, BChE, acylpeptide hydrolase, and carboxylesterase have a conserved active site serine with the consensus sequence GXSXG. When the active site serine is covalently modified by OP, the enzyme loses activity. Loss of enzyme activity allows one to conveniently measure reactivity with OP.

Proteins with no catalytic activity are a novel class of OP target proteins. They have no active site serine and have been

shown to bind OP *in vitro*, but not in living animals. The advent of quadrupole time-of-flight mass spectrometry has made it possible to positively identify albumin as a protein that binds the OP FP-biotin in living mice.

Experiments with purified proteins have documented covalent attachment of OP not only to serine, but also to tyrosine and histidine. For example, human albumin covalently binds sarin, soman, and DFP at tyrosine (Black *et al*., 1999; Means and Wu, 1979). Bovine serum albumin is readily phosphorylated by DFP with a stoichiometry of one DFP molecule bound per molecule of albumin (Murachi, 1963). The sequence of the albumin peptide that covalently binds DFP was reported by Sanger as ArgTyrThrLys for human and rabbit albumin, and ArgTyrThrArg for bovine albumin (Sanger, 1963). After the complete amino acid sequences of the albumins was known, these reactive tyrosines were identified as Tyr 411 in human and Tyr 410 in bovine albumin (Meloun *et al*., 1975; Peters, 1996). Papain binds DFP on tyrosine (Chaiken and Smith, 1969), while rabbit liver carboxylesterase binds DFP on histidine as well as on the active site serine (Korza and Ozols, 1988). Bromelain is not inhibited by DFP, but it does react with DFP, leading to the formation of a fully active, phosphorus-containing enzyme (Murachi, 1963; Murachi *et al*., 1965). The rat M2 muscarinic acetylcholine receptor covalently binds chlorpyrifos oxon, though the binding site has not yet been identified (Bomser and Casida, 2001; Huff *et al*., 1994).

Living animals have previously been demonstrated to bind OP to noncholinesterase sites. The tissue distribution of radiolabeled DFP in rabbits (Jandorf and McNamara, 1950), and radiolabeled soman in rats and mouse brain (Little *et al*., 1988; Traub, 1985) had no correlation with cholinesterase localization. These results have been interpreted to mean that tissue proteins other than cholinesterase are capable of binding OP poisons (Jandorf and McNamara, 1950; Kadar *et al*., 1985).

Existing Mass Spectrometric Methods for Diagnosis of OP Exposure

Gas chromatography combined with mass spectrometry was used to detect sarin in archived blood samples from victims of the 1995 Tokyo subway attack (Polhuijs *et al*., 1997). The covalently bound sarin in 0.12 to 0.5 ml of serum was released by incubation with 2 M potassium fluoride at pH 4.0. This method confirmed that the people had been exposed to OP and, in addition, identified the OP as sarin. The mass-to-charge ratios of the released OP fragments were 81, 99, and 125, values characteristic of sarin.

The GC-MS method is a significant advance over simple inhibition assays, but it has limitations. (1) The method relies on being able to release the OP from its covalent attachment site on BChE with potassium fluoride. The release step requires the catalytic machinery of BChE to be intact. Samples that have been stored in ways that denature the BChE cannot undergo fluoride-induced release of OP (Polhuijs *et al*., 1997). (2) Proteins other than BChE, AChE, and carboxylesterase may not be able to release OP upon treatment with potassium fluoride. Human albumin after phosphylation with sarin is not amenable to reactivation with fluoride ions (Van Der Schans *et al*., 2004). (3) OP-derivatized protein samples that have lost an alkyl group from the phosphonate in the process called 'aging' would not be capable of releasing their OP upon treatment with potassium fluoride.

GC-MS of OP metabolites is the method recommended by the Centers for Disease Control for monitoring potential exposure to nerve agents. Measurement of OP metabolites is limited by the fact that these compounds are rapidly cleared from the body. Sarin metabolites were found in urine on post-exposure days 1 and 3, and in trace amounts on day 7 in a man who had inhaled a dose that made him unconscious (Nakajima *et al*., 1998). Urine samples from four patients hospitalized for sarin exposure showed that most of the sarin metabolites had cleared within 24 h (Hui and Minami, 2000).

Many of these limitations were overcome in an approach where plasma BChE was purified by affinity chromatography and digested with pepsin. The peptides were separated by liquid chromatography, and the OP-derivatized peptide as well as the OP it carried were identified by electrospray tandem mass spectrometry (Fidder *et al*., 2002). This method is well suited to detect OP exposure and will become even more valuable if OP-derivatized proteins other than BChE are included in the analysis. A major advantage of using protein-bound OP to diagnose exposure is that the OP-derivatized proteins remain in the circulation for weeks (Cohen and Warringa, 1954; Grob *et al*., 1947; Munkner *et al*., 1961; Van Der Schans *et al*., 2004).

Albumin Properties

Albumin is a nonglycosylated single chain of 585 amino acids folded into three domains by 17 intrachain disulfide bonds. Albumin is the most abundant soluble protein in the body of all vertebrates and is the most prominent protein in plasma. It is synthesized in the liver and discharged into the bloodstream. Of the 360 g total albumin in a human, 40% resides in the blood and 60% in extravascular fluids of tissues (Peters, 1996). The rate of transfer to the extravascular space is about 4.5% per hour. About 40% of the extravascular albumin is in muscle. Albumin is responsible for the colloid osmotic pressure of plasma and for maintenance of blood volume and supplies most of the acid/base buffering action of plasma proteins in extravascular fluids. Albumin binds and transports metabolites and drugs. Analbuminenia, a rare deficiency of albumin, is compatible with nearly normal health in humans. The study of analbuminemia shows that albumin is helpful in coping with stress and in containing environmental and physiological toxins.

Albumin and BChE are Attractive Biomarkers for OP Poisoning

Both albumin and BChE are found in human plasma, a tissue that can be readily sampled for biomarkers of OP exposure. Both proteins bind OP, though the binding to albumin of OP other than FP-biotin has not been demonstrated *in vivo*. Both proteins have a long half-life in the circulation of humans, 20 days for albumin (Chaudhury *et al*., 2003; Peters, 1996) and 10–16 days for BChE (Cohen and Warringa, 1954; Jenkins *et al*., 1967; Munkner *et al*., 1961; Ostergaard *et al*., 1988). The long half-life of OP-labeled proteins contrasts with the short half-life of OP and OP metabolites in urine. This provides a significant advantage to a method that uses OP-labeled proteins for diagnosis of OP exposure.

Role of Albumin in OP Toxicity

Binding of OP to albumin could serve to scavenge OP molecules and therefore reduce the amount of OP available for reaction with AChE. The affinity of various OP for albumin is likely to vary, so the effectiveness of albumin as an OP scavenger is likely to depend on the identity of the OP.

Another consideration in evaluating the role of albumin in OP toxicity is competition with drugs for binding to the same site in albumin. Several drugs, including diazepam (Fehske *et al*., 1979; Peters, 1996) and ibuprofen, bind to the same region of albumin that binds OP (see Tables 3–5 in Peters, 1996). Since the free form of a drug is active, the extent of binding to albumin controls both the effect and duration of the drug. Decreased binding of a drug to albumin can cause toxicity owing to an increase in the concentration of its free form. Thus, a person being treated for intestinal worms with metrifonate (an OP that is converted nonenzymatically to dichlorvos) might be unable to tolerate a standard dose of diazepam or ibuprofen. Or conversely, a person taking ibuprofen, could be intolerant of a standard dose of metrifonate.

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Fast Affinity Purification Coupled with Mass Spectrometry for Identifying Organophosphate Labeled Plasma Butyrylcholinesterase

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Abstract

Classical plasma butyrylcholinesterase (BChE) purification involves dialysis and multiple steps of chromatography. We describe here a procainamide affinity gel purification scheme that takes 30 minutes to purify BChE from 1 ml of plasma. The method uses a microfuge filter tube to build a 0.2 ml procainamide affinity column. The eluted BChE contains 3-4 µg of 500-fold purified BChE, free from 99% of contaminating plasma proteins. The BChE was further purified by

gel electrophoresis. Tryptic peptides from the BChE containing gel electrophoresis band were prepared by in-gel digestion, separated by reverse phase liquid chromatography and identified by mass spectrometry. The 29 residue active site tryptic peptide labeled with a nerve agent either soman or sarin was identified.

Introduction

Butyrylcholinesterase (BChE) is a scavenger protein that protects the cholinergic system against anticholinesterase poisons (1, 2). Most of the U.S. population has been exposed to organophosphorus (OP) pesticides in their homes, workplaces, outdoors, or through trace contaminants in food (3). The high reactivity of BChE with OP makes BChE an ideal biomarker of OP exposure. OP inhibits BChE by covalently binding to its active site serine. Proteolysis of OP-inhibited BChE generates an OP-peptide conjugate whose molecular mass distinguishes nerve agents from OP pesticides. Mass spectrometry analysis of the plasma BChE peptide can be used to determine whether a person was exposed to OP and what kind of OP he or she was exposed to (4, 5, 6).

The 12 most abundant proteins in plasma make up 96% (by weight) of the total plasma proteins. Their concentrations range from 50 mg/ml (albumin) to 1 mg/ml (apolipoprotein A) (7). Human BChE concentration in blood is 4.2×10^{-3} mg/ml. Even though mass spectrometry technologies have advanced to high

sensitivity, purification steps are still necessary to identify a low abundant protein like BChE in a complex plasma sample (8).

Here we describe a fast and simple affinity purification method for plasma BChE that produces BChE sufficiently pure that it can be identified by mass spectrometry. The purification procedure consumes as little as 1 ml of plasma. A similar procedure has been described by Fidder and coworkers (5). However, our purification is substantially simpler and has the potential to be developed into a high throughput method. The procedure was applied to samples of human plasma treated with the nerve agents soman and sarin. Peptides from BChE, isolated from 1 ml of plasma, were identified including the OP-labeled active-site peptide. The identity of the OP-labeled active site serine peptide was confirmed by MS/MS spectrum.

Experimental Methods

OP treatment of human plasma: Human plasma was treated with 200 μM soman or sarin, reducing BChE activity to zero. Soman and sarin were provided by CEB (Vert-le-Petit, France). Only trace amounts of intact soman and sarin remained in the plasma after 24 hours at room temperature. Samples were stored at -80˚C. Plasma was cleared of solids and fat by centrifugation.

Procainamide-Sepharose micro column purification of plasma BChE: Procainamide-Sepharose gel, custom made by Dr. Yacov Ashani (9), bound 34 μmoles of procainamide per ml gel. 0.2 ml (0.4 ml of 1:1 slurry in 50% ethanol)

of procainamide gel was packed into a 1.5 ml microfuge spin column (Princeton Separations, Adelphia, NJ). The column was equilibrated with 2 ml of 20 mM potassium phosphate pH 7.0 buffer. 1 ml of cleared, OP-treated plasma was allowed to flow through the column by gravity flow at a flow rate of 1 ml/10 minutes. The column was washed 4 times with 1 ml of 0.2 M NaCl in 20 mM potassium phosphate pH 7.0 buffer. Each wash time was reduced to less than 1 minute by briefly centrifuging the column. BChE was eluted with 0.5 ml of 1 M sodium chloride in 20 mM potassium phosphate pH7.0 buffer or alternatively with 0.5 ml of 0.2 M procainamide.

Nondenaturing gradient gel electrophoresis: A four-to-thirty percent, polyacrylamide, nondenaturing, gradient gel, 0.75 mm thick, was prepared in a Hoefer SE6000 gel apparatus (Hoefer Scientific Instruments, San Francisco, CA; presently part of Pfizer Inc). Electrophoresis was at 250 V constant voltage for 16 hours at 4°C. 10 µl-samples were mixed with 50% glycerol in 0.1 M Tris/Cl, pH7.5 to a final glycerol concentration of 10%. The gel was first stained for BChE activity using the Karnovsky & Roots method (10), and then stained with Coomassie blue R-250 (Fisher Scientific).

The BChE intended for mass spectrometry was reduced from 0.5 ml to 60 ul and desalted in an Amicon YM10 centrifugal filter with a molecular weight cutoff of 10K Da (Millipore, Billerica, MA) before it was loaded on the nondenaturing gel. A nondenaturing gel rather than an SDS gel was used because only the nondenaturing gel separates BChE from albumin.

Protein in-gel digestion and peptide extraction: The protein band corresponding to the position of BChE was cut out and digested with trypsin (11). Peptides were extracted from the gel and dissolved in 5% acetonitrile, 0.1% formic acid for mass spectrometry analysis.

ESI LC-MS/MS analysis of tryptic peptides: A FAMOS autosampler in conjunction with a SWITCHOS and ULTIMATE capillary liquid chromatography system (LC Packings Dionex, Sunnyvale, CA) was used to deliver peptides to a QTrap hybrid quadrupole, linear ion trap mass spectrometer model 2000 (Applied Biosystems, Foster City, CA). Peptides were eluted from a Vydac C18 nanocolumn (Grace Vydac, Southborough, MA) at a flow rate of 300 nl/min, using an acetonitrile gradient containing 0.1% formic acid. The acetonitrile increased from 5 to 60% in 60 minutes. Detailed instrument settings and data collection protocols are described by Schopfer et al (12). MASCOT (Matrix Science, Boston, MA) was used for database searching to identify proteins (13).

Results and Discussion

A single-step procainamide affinity purification recovers 70% of the starting BChE.

1 ml of control plasma was applied onto a 0.2 ml procainamide-sepharose column packed into a microfuge spin column. As shown in table 1, 94% of the BChE was retained on the column and 70% of the starting BChE was recovered

by elution with 1 M NaCl. The time elapsed from loading the sample to elution was 30 minutes.

Table 1. Procainamide affinity purification of BChE from 1 ml plasma.

Fraction	composition	volume, ml	BChE activity, u/ml
Loading	plasma	1	2.95
flow-thru	plasma eluted during loading	1	0.17
wash-off	0.2 M NaCl	1	0.17
	0.2 M NaCl	1	0.06
	0.2 M NaCl	1	0.04
	0.2 M NaCl	1	0.03
elute	1 M NaCl	0.5	4.15

OP labeled BChE tryptic peptide identified from gel extracts by LC-MS/MS.

BChE from 1 ml of soman- or sarin-treated human plasma was subjected to the purification procedure described above. Protein eluates from the procainamide column were loaded onto a nondenaturing gel. The gel was first stained for BChE activity, then counter stained with Coomassie blue. Figure 1A shows the chromatography results for sarin-treated samples. Relatively strong BChE activity staining bands can be seen on the gel (Figure 1A, lanes 2, 3, 4), indicating that plasma BChE activity had partially recovered. Comparison of the Coomassie staining in Figure 1B, lane 1 (unpurified plasma) and lane 4 (eluate from the column), shows that the one-step procainamide purification eliminated significant amounts of high abundant proteins including albumin. Although the

preparation still contained other proteins (Figure 1B, lane 6), the sample was pure enough for mass spectrometry identification of BChE.

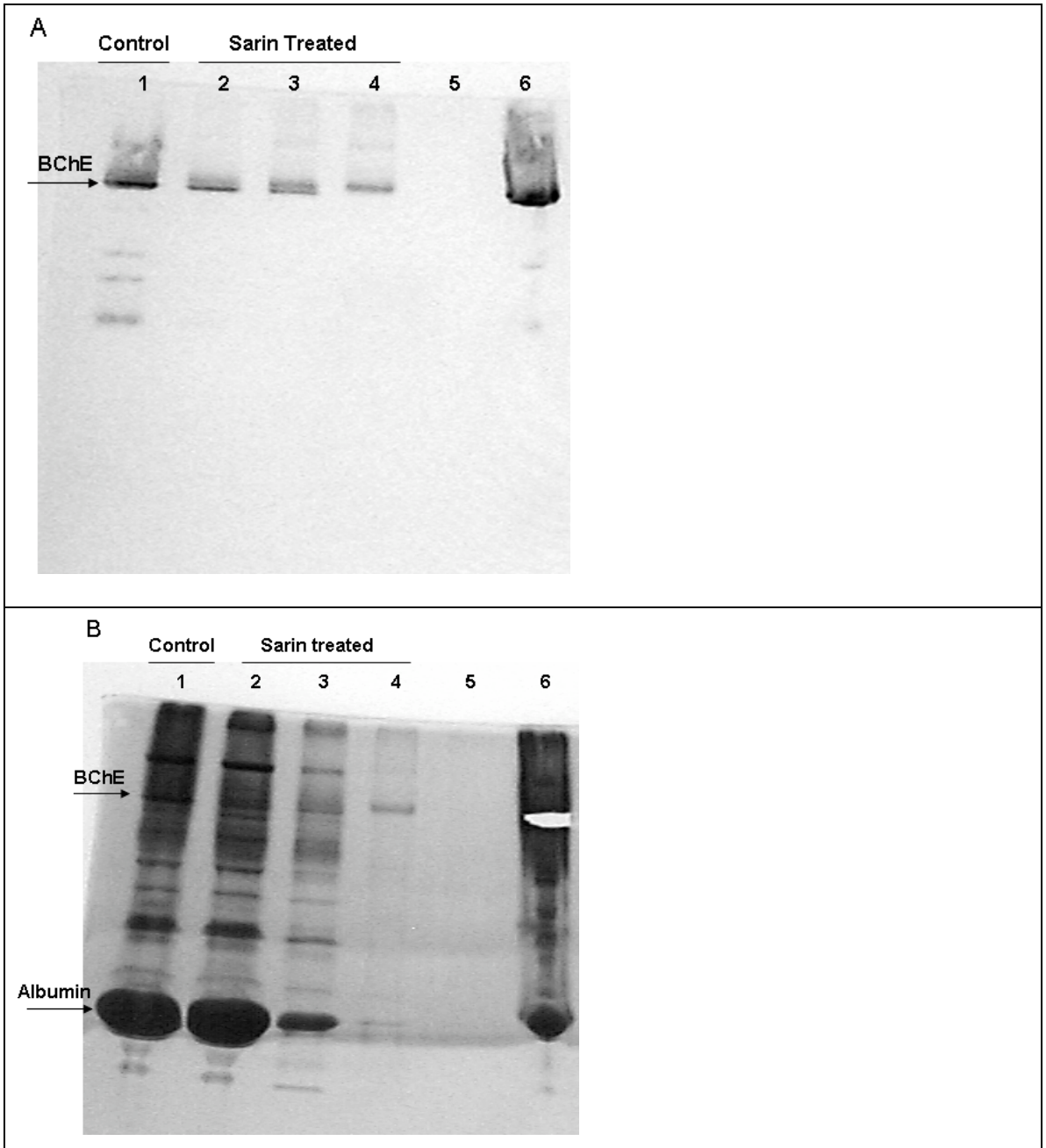

Figure 1. BChE activity (A) followed by Coomassie blue staining (B) on a nondenaturing gel of sarin-treated, plasma proteins from the procainamide affinity column. Lane 1: control human plasma (no sarin treatment, no purification); Lane 2: flow-through fraction from sarin-treated

plasma that did not bind to the affinity column; Lane 3: wash-off fraction; Lane 4: eluate from the affinity column; Lane 5: blank; Lane 6: concentrated eluate. The gel slice used for mass spectrometry was cut out of lane 6. Equal volumes (10 ul) of sample were loaded in lanes 1-4. Note that significant amounts of protein were in the flow-through fraction.

Table 2 summarizes proteins identified from the concentrated BChE band in lane 6 of the gel. BChE was found as the third most prominent protein. It had a Mowse score of 561. 14 peptides were identified, covering 34% of the BChE sequence. 6 peptides scored in the identity range, 7 in the homology range, and 1 below homology. The soman/sarin-aged, active-site BChE peptide was among those identified. It appeared as a quadruply-charged ion at 752.9 m/z. Manual inspection of the MS/MS spectrum confirmed its identity (data not shown).

Table 2. Human proteins that co-migrate with BChE on nondenaturing gel, identified by LC-MS/MS and MASCOT database search.

Accession number [a]	Protein name [b]
IPI00783987	Complement component 4B preprotein
IPI00418163	Complement component C3 precursor
IPI00025864	BChE
IPI00021891	Isoform Gamma-B of fibrinogen gamma chain precursor
IPI00305461	Inter-alpha-trypsin inhibitor
IPI00298971	Vitronectin precursor
IPI00784810	IGHV4-31 protein
IPI00829944	IGHG1 protein

IPI00298497	Fibrinogen beta chain precursor
IPI00292530	Inter-alpha-trypsin inhibitor heavy chain H1 precursor
IPI00032328	Isoform HMW of kininogen-1 precursor
IPI00784070	IGKC protein
IPI00430808	IGKC protein
IPI00021885	Isoform 1 of fibrinogen alpha chain precursor
IPI00021842	Apolipoprotein
IPI00294193	ITIH4 isoform 1 of Inter-alpha-trypsin inhibitor
IPI00168728	FLJ00385 protein (fragment)
IPI00019576	Coagulation factor 10 precursor
IPI00550640	IGHG4 protein
IPI00399007	putative uncharacterized protein

[a]: Data analysis was performed using MASCOT with the International Protein Index (IPI) database (http://www.ebi.ac.uk/IPI/IPIhelp.html).

[b]: Proteins listed are top 20 hits from the MASCOT search with lowest Mowse score being 75. Individual ions scores > 40 indicate identity or extensive homology ($P<0.05$).

Soman treated plasma had no BChE activity to pinpoint the location of the labeled BChE on the gel. However, removal of a band from a location adjacent to BChE in the control lane provided a sample from which the tryptic peptides of BChE could be identified by LC-MS/MS. MASCOT identified the aged, soman-labeled, BChE active-site peptide as a quadruply charged parent ion of 752.7 m/z. The aged products of soman- and sarin-inhibited BChE are expected to

have the same mass (14). Manual inspection of the MS/MS spectrum of peptide ion 752.7 confirmed the MASCOT assignment (Figure 2).

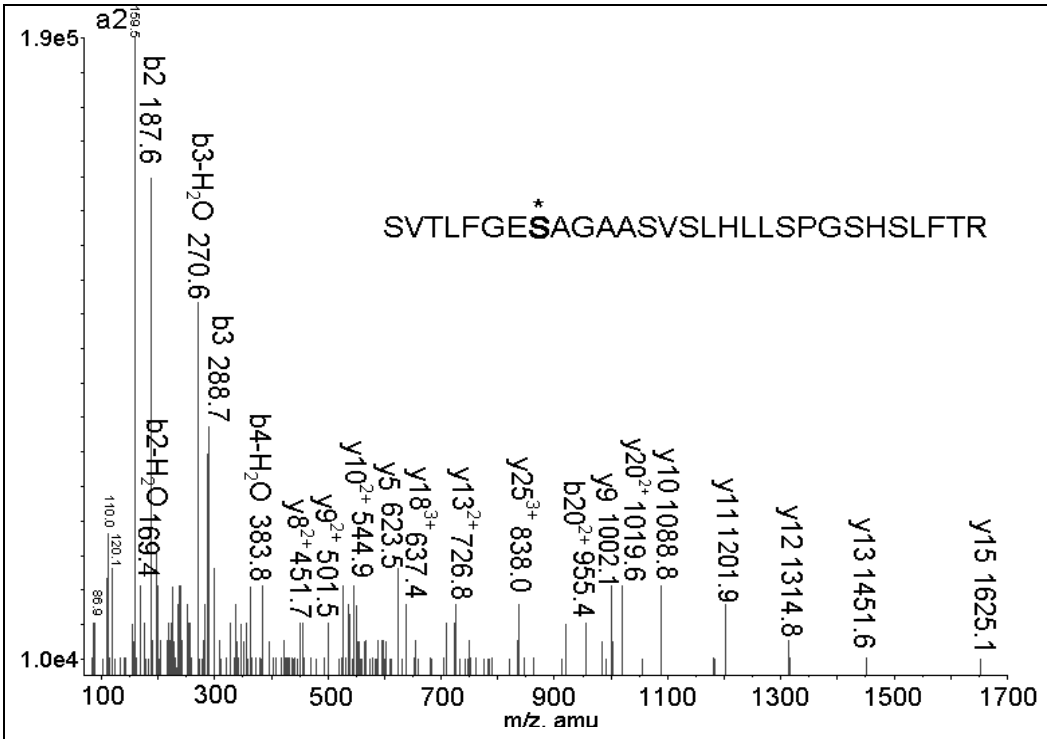

Figure 2. MS/MS spectrum of the aged, soman-labeled BChE active-site peptide. The sequence of the BChE active-site tryptic peptide is shown. The active-site serine is labeled with *. The experimental m/z of the quadruply charged parent ion was 752.7.

The methods developed in this report can be used to analyze plasma not only for nerve agent exposure, but also for organophosphorus pesticide and carbamate exposure.

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